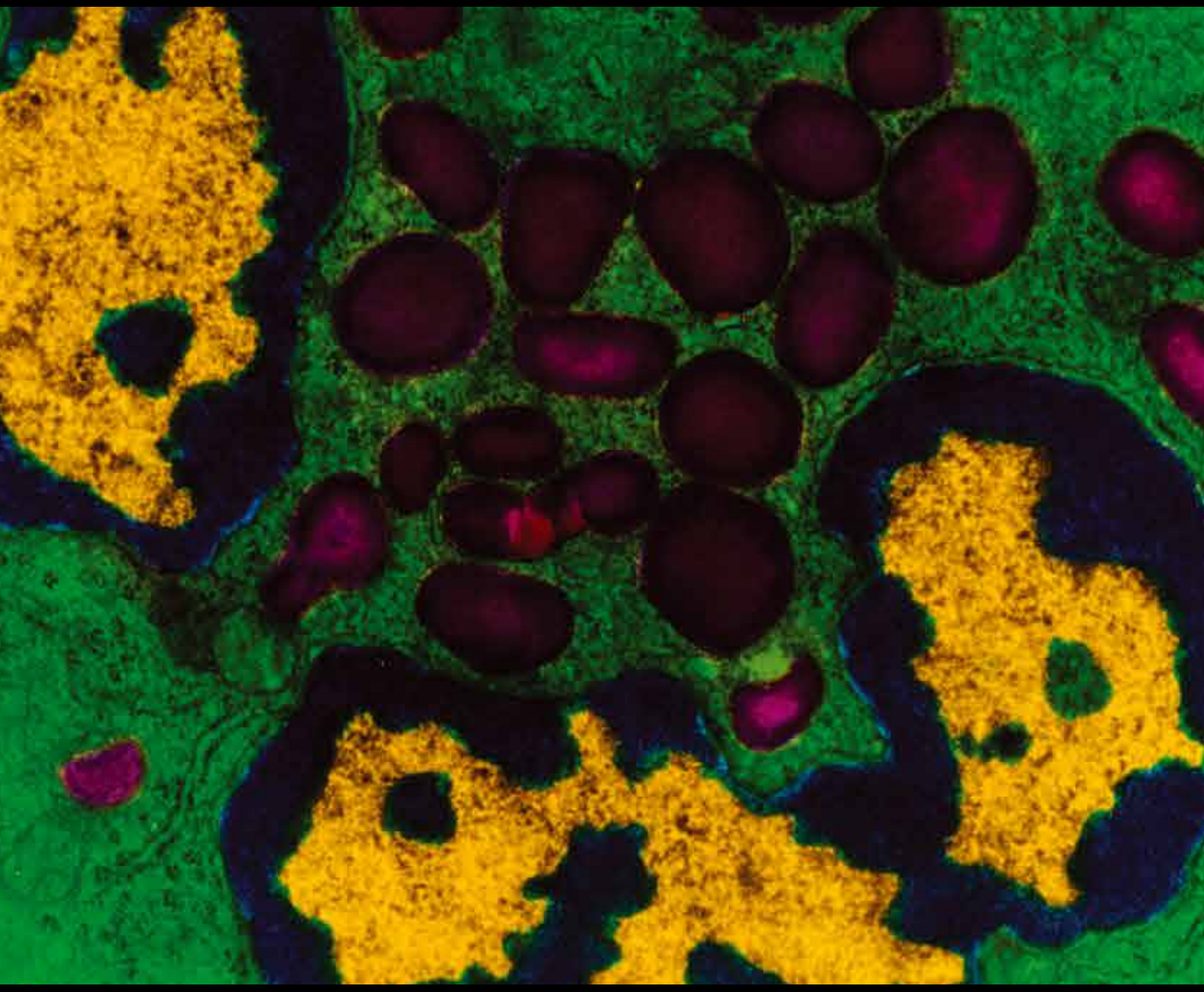
Mediators of Inflammation and Immune Responses in the Human Gastrointestinal Tract

Guest Editors: E. Arranz, A. S. Peña, and D. Bernardo



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Editorial Board

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 865638, 3 pages
http://dx.doi.org/10.1155/2013/865638

Editorial

Mediators of Inflammation and Immune Responses in the Human Gastrointestinal Tract

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Received 21 July 2013; Accepted 21 July 2013

The gastrointestinal tract is continuously exposed to foreign antigens—mainly derived from the commensal microbiota and food antigens—but occasionally to those derived from invading bacteria, viruses, and tumoral antigens. Therefore, the immune system of the gut has a unique capacity to balance the mechanisms of tolerance in health and those creating a proper defensive immune response in disease. Changes in such delicate balance are usually linked to the development of gastrointestinal pathology. Despite its central role in human health and disease, most of the current knowledge of mucosal immunology of the gastrointestinal tract is mainly obtained from experimental murine models. Although the mechanisms of intestinal immunity in mouse and human have similar output, the specific pathways through which they are elicited are different [1, 2]. It is essential to fill in this gap in our current knowledge of the human immune system of the gastrointestinal tract in order to understand the pathogenesis and be able to design rational therapies to manage acute and chronic inflammatory gastrointestinal disease. In this special issue, we aimed to gain depth into the current understanding of immune processes in the human gastrointestinal tract in health and disease by selecting work in progress of active investigators in the field.

L. Pastorelli et al. review the role of a new cytokine IL-33, member of the IL-1 family, in promoting host defense against parasite and involved in the pathogenesis of ulcerative colitis. The authors discuss some contradictory reports on IL-33 function in the gastrointestinal mucosa, where it has been reported either to enhance inflammatory responses or to promote epithelial integrity [3, 4].

Significant advances have been achieved in the understanding of the pathogenesis of inflammatory bowel disease (IBD), and new therapy targets are cytokines as well as their receptors and signaling pathways [5]. In this issue, F. Scaldaferri et al. review the several immune factors taking part in inflammatory bowel disease and how they are modulated in the course of therapy aiming to identify potential targets to control. These authors have confidence that new emerging techniques, like microarray analysis or miRNA analysis, which are able to assess immune signatures in response to therapy, could help to identify good candidates for mucosal prognostic biomarkers, together with new therapeutic targets for future research.

Vascular endothelial growth factor (VEGF) is a predominant angiogenic factor, and recent studies using tissue microarray blocks of VEGF-A, VEGF-C, VEGFR-2, and VEGFR-3 expression have shown an association to progression, invasion, and metastasis leading to poorer survival rates and prognosis [6]. G. Karamanolis et al. show an increased expression of both VEGF and CD31 in postradiation rectal biopsy specimens, suggesting that the blockage of VEGF may represent a therapeutic option in patients with these severe conditions that is refractory to available therapies.

Recent works suggest that in coeliac disease both the innate and the acquired immune response are involved in the inflammation initiated and maintained by gluten and key to the development of autoimmunity in this common disease [7]. A small fraction of patients become refractory to the gluten-free diet, the only current available treatment [8, 9]. Recent studies suggest that it is more frequently observed

in Europe than in the United States [10]. It is characterized by persistent or recurrent symptoms of malabsorption and intestinal villous atrophy. In this issue, S. Gross et al. have studied refractory CD type II (RCDII), a particular subtype with extreme bad prognosis and in fact it is considered a low-grade intraepithelial lymphoma [11]. Gross et al. have found that IL-13 may play a key role as a proinflammatory cytokine since it is correlated with IL-17A production and to other TH1 and TH2 cytokines, but not to the regulatory cytokine IL-10, thus confirming their hypothesis that the immune response is differentially regulated by cytokines in active coeliac disease versus RCDII. This finding opens new mechanisms to study further in order to understand the pathogenesis of this condition.

Obesity, the modern epidemics, is according to a new vision associated with chronic low-grade inflammation, and the intestinal microflora may be responsible for inducing these changes [12]. Since fecal microbiota transplantation re-establishes a balanced intestinal flora with resultant cure of recurrent clostridium difficile infection [13], other conditions may benefit from this approach. In this issue, R. Mehta et al. have studied gene expression profile in gastric tissue of morbidly obese patients with different histological forms of nonalcoholic fatty liver disease (NAFLD) and have identified an altered profile for several inflammatory molecules. This finding may be responsible for the pathogenesis of obesity-related NAFLD. Previously, it had been demonstrated that alterations in intestinal microbiota are associated with obesity and six weeks after infusion of microbiota from lean donors increased insulin sensitivity of recipients along with levels of butyrate-producing intestinal microbiota. Thus suggesting that intestinal microbiota might be developed as therapeutic agents to increase insulin sensitivity in humans, however, increased knowledge of the intestinal microbiota in health maintenance as well as controlled trials of fecal microbiota transplantation is needed before it can be accepted to be used clinically [14, 15].

The activation of several cytosolic pathogen recognition receptors allows the assembly of the inflammasome, a multimeric complex platform that leads to the activation of the innate immune system [16]. In their paper presented in this issue, T. Nunes and H. S. de Souza review our current knowledge on the inflammasome. The known molecular structure, its importance in maintaining intestinal homeostasis, and its critical mechanisms of the inflammasome are described in the context of chronic inflammatory disorders in the human gut such as inflammatory bowel diseases (IBD) and intestinal cancer.

M. Witkowska and P. Smolewski, in this issue, review the role of *Helicobacter pylori* infection in chronic inflammation and the subsequent genomic transformation and development. Knowledge on the etiology, pathogenesis, treatment, and follow-up of gastric mucosa-associated lymphoid tissue (MALT) lymphoma is providing more insight into mechanisms of inflammation of this infection that is fortunately continuously decreasing in the western world. *Helicobacter pylori* eradication is a first-line treatment of gastric MALT lymphoma; however, a significant percentage of patients do not respond to treatment. Recently, it has been found that a high number of Treg cells or a high ratio of Treg cells to the total number of CD4+ T cells in gastric MALT lymphoma could predict responsiveness to eradication therapy [17].

Manipulation of gut microbiota composition by using probiotics is being explored as a promising avenue of prophylactic and therapeutic intervention against gut inflammation. Current evidence provides support for the consideration of probiotics therapy for intestinal diseases, keeping in mind that efficacy of probiotics is strain and disease specific. The variety of studies carried out with distinct strains of probiotics bacteria has suggested heterogeneous and strain-specific effects. Because of the limitations of most studies conducted with probiotics, with regard to the power of the study, deficit of human studies, randomization, use of different strains, and lack of standardized methodology, it remains difficult to draw firm conclusions from the current trials [18]. In this issue, R. Sengupta et al. discuss the role of cell surface-associated molecules in the probiotics and their host receptor. These mechanisms will help to have a better understanding of the probiotics-host crosstalk and contribute to improve therapies to treat or prevent gastrointestinal inflammation in IBD.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most highly prescribed drugs in the world for the treatment of pain, inflammation, and fever. However, these drugs produce serious gastrointestinal complications during long-term administration, particularly in the elderly [19]. M. Sinsa et al., in this issue, describe the action of NSAIDs as a cause of morbidity/mortality related to gastric and duodenal ulcer disease and discuss different approaches to prevent or minimize such adverse effects. However, they caution that these treatments are effective to some extent, but most of them are also associated with other risks, and there is a need to develop novel therapeutic agents to make the use of NSAIDs safer.

Particular probiotics of the *Lactobacillus* species appear to stimulate health promoting effects in the gastrointestinal tract, such as pathogen inhibition by competing with invading bacteria, immunomodulation, and enhancement of the epithelial integrity [20] not only via direct contact but also through bacteria derived metabolites [21]. Recent works suggest that dendritic cells (DC) control the nature and location of immune response that seems to play a central role in ulcerative colitis [22]. Dendritic cells may orchestrate the abnormal response against the commensal microbiota that is present in these patients [23]. E. R. Mann et al. describe an abnormal phenotype and function of circulating DC in patients suffering from ulcerative colitis which are partially restored by the probiotic strain *Lactobacillus casei Shirota*.

It has been a pleasure to select the work presented in these areas by experts in the respective fields. We hope that their findings will help to enrich the knowledge of the mediators of inflammation of the human gastrointestinal tract and will form the basis for new approaches to the treatment of common infections and those conditions that although rare have such a bad prognosis.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 573576, 12 pages
http://dx.doi.org/10.1155/2013/573576

Research Article

Dysregulated Circulating Dendritic Cell Function in Ulcerative Colitis Is Partially Restored by Probiotic Strain *Lactobacillus casei* Shirota

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Received 4 February 2013; Revised 29 May 2013; Accepted 30 May 2013

Academic Editor: Eduardo Arranz

Background. Dendritic cells regulate immune responses to microbial products and play a key role in ulcerative colitis (UC) pathology. We determined the immunomodulatory effects of probiotic strain *Lactobacillus casei* Shirota (LcS) on human DC from healthy controls and active UC patients. *Methods*. Human blood DC from healthy controls (control-DC) and UC patients (UC-DC) were conditioned with heat-killed LcS and used to stimulate allogeneic T cells in a 5-day mixed leucocyte reaction. *Results*. UC-DC displayed a reduced stimulatory capacity for T cells ($P < 0.05$) and enhanced expression of skin-homing markers CLA and CCR4 on stimulated T cells ($P < 0.05$) that were negative for gut-homing marker $\beta 7$. LcS treatment restored the stimulatory capacity of UC-DC, reflecting that of control-DC. LcS treatment conditioned control-DC to induce CLA on T cells in conjunction with $\beta 7$, generating a multihoming profile, but had no effects on UC-DC. Finally, LcS treatment enhanced DC ability to induce TGFβ production by T cells in controls but not UC patients. *Conclusions*. We demonstrate a systemic, dysregulated DC function in UC that may account for the propensity of UC patients to develop cutaneous manifestations. LcS has multifunctional immunoregulatory activities depending on the inflammatory state; therapeutic effects reported in UC may be due to promotion of homeostasis.

1. Introduction

Interactions between the host and microbiota play a crucial role in mucosal immune homeostasis [1]. Certain strains of lactic-acid producting bacteria are classed as probiotics because their consumption is associated with health benefits, which are mediated via the gut. The current probiotic definition is "live microorganisms which when administered in adequate amounts confer a healthy benefit on the host" [2]. Probiotic bacteria are most frequently of the *Lactobacillus* or *Bifidobacterium* species, and usually species that can be found in the normal commensal microbiota. Probiotics can be effective in treating some patients with inflammatory bowel disease (IBD) [3–7] but the details of which strains confer benefit and their mechanisms of action are only slowly being defined.

Ulcerative colitis (UC) and Crohn's disease (CD), collectively termed inflammatory bowel disease (IBD), result from a dysregulated response of the mucosal immune system to components of the luminal microbiota, and breakdown of immune tolerance in individuals who are genetically predisposed to the disease These processes lead to "inappropriate" activation of mucosal T-cells and production of inflammatory mediators [8–11].

Dendritic cells (DC) recognize and respond to bacteria and bacterial products, and generate primary T-cell responses. DC also determine whether T-cell responses generated are immunogenic or tolerogenic [12–14]. In particular, intestinal DC maintain the delicate balance in the gut between immunogenicity against invading pathogens and tolerance of the commensal microbiota [15]; alterations in intestinal DC have been found in IBD [15, 16]. The effects of probiotic bacteria on DC, which are so pivotal in early bacterial recognition, tolerance induction and shaping T-cell responses, are likely to be central in immunomodulation by these bacteria, and are likely to partially account for the reported efficacy of probiotics in IBD [3–7].

IBD is associated with a variety of extra-intestinal manifestations (EIM), with up to a third of IBD patients developing cutaneous manifestations [17]. The causes of EIM are poorly understood, but it has been suggested that compartmentalisation of inflammatory processes to different organs (e.g., the intestines, skin or liver) may be linked to homing and trafficking of immune cells [18]. Indeed, dysregulated lymphocyte trafficking has been reported in both UC and CD [19–22].

Homing properties are imprinted on T-cells upon stimulation by DC, to localise immune responses to specific tissues [23–26]. Effector T-cells migrating to intestinal sites express high levels of gut-homing molecule $\alpha_4\beta_7$ [27], with its ligand MAdCAM-1 being constitutively expressed by post-capillary endothelial cells in the small intestine [28] and colonic lamina propria [29]. Skin T-cells express E- and P-selectin ligands including cutaneous lymphocyte-associated antigen (CLA) [30], and CCR4 [31]. The occurrence of EIM associated with IBD indicates a systemic disease, rather than immune dysregulation confined to intestinal sites; however it is currently unclear whether alterations in *circulating* DC occur in IBD patients, including DC ability to imprint specific homing properties on stimulated T-cells. Trafficking of immune cells is an area yet to be investigated regarding specific mechanisms of action of immunomodulation by probiotics or dysregulated DC function in IBD.

The strain-specific nature of the immunomodulatory effects of probiotics is well established; some *Lactobacillus* strains induce production of regulatory cytokines, suppress Th1 responses and are thought to be involved in oral tolerance. In contrast, other strains induce production of pro-inflammatory cytokines. However, human intervention studies have shown a variety of beneficial immunomodulatory effects associated with consumption of the probiotic bacterial strain *Lactobacillus casei* Shirota (LcS) specifically, including significant improvement in UC disease activity index (UCDAI) scores in patients with mild-moderate UC administered LcS orally for 8 weeks, compared to pre-treatment and also patients on conventional therapy. The same study demonstrated LcS reduces production of IL-6 from peripheral blood mononuclear cells (PBMC) *in vitro* [32]. Other studies demonstrate reduction of gingival inflammation [33], and downregulation of allergic responses [34] following consumption of LcS. To this end, we aimed to determine whether systemic changes exist between healthy controls and patients with active UC, regarding the ability of circulating (blood-enriched) DC to generate effector T-cell responses and imprint specific homing properties on T-cells stimulated. We also aimed to study the immunomodulatory effects of probiotic strain LcS on such DC.

2. Materials and Methods

2.1. Human Peripheral Blood. Human peripheral blood was collected from healthy volunteers with no known autoimmune or inflammatory diseases, allergies or malignancies ($n = 8$), or from patients with active UC following informed consent ($n = 6$). Disease activity for UC was assessed using the UC disease activity index (UCDAI); patients scoring UCDAI 4–12, alongside diagnosis from clinical parameters, radiographic studies, endoscopic and histological criteria, were defined as active UC. Patients were treatment naïve or on minimal treatment: 5-aminosalicylic acid (5ASA) and/or azathioprine (AZA). Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation over Ficoll-Paque plus (Amersham Biosciences, Chalfont St. Giles, UK). Human blood-enriched DC (low density cells or LDC) were obtained following NycoPrep centrifugation of overnight cultured PBMC. These cells were 98–100% HLA-DR$^+$, with morphological characteristics of DC (both at optical microscopy and electron microscopy), and are potent stimulators of naïve T-cells. Blood LDC have been characterised in detail in previous studies from our laboratory [35, 36], and will be referred to as blood DC in this study.

2.2. Conditioning of Human Blood DC by LcS. Stock culture of LcS (Yakult Honsha Co. Ltd., Tokyo, Japan) were cultured at 37°C for 24 hours in MRS broth and grown on MRS agar (Oxoid, Hampshire, UK) for 48 hours at 37°C in an anaerobic cabinet (MACS MG 1000; Don Whitley Scientific, West Yorkshire, UK) with a gas mixture of 10% H_2, 10% CO_2 and 80% N_2 by volume. For liquid culture, one pure colony was taken from an MRS nutrient agar plate and grown overnight in 10 mL of pre-reduced MRS broth (Oxoid) with 0.05% L-cysteine hydrochloride (Sigma, Dorset, UK) in a shaking incubator at 37°C; 0.5 mL of the overnight culture was inoculated into another 10 mL MRS broth. The bacteria were harvested in the exponential phase, resuspended in phosphate-buffered saline (PBS; Oxoid), centrifuged twice at 1960 g (Sanyo/MSE Micro Centaur, Haverhill, USA) for 5 minutes and resuspended at the required concentration in RPMI 1640 containing 0.75 mM L-glutamine. Bacteria were then heat-killed with viability checks done to make sure no bacteria survived, and varying concentrations (1×10^5, 1×10^6, or 1×10^7) of heat-killed LcS were used to condition 2.5×10^5 blood DC in 1 mL total volume of complete medium (Dutch modification RPMI 1640 containing 2 mM glutamine, 10% fetal calf serum and 100 U/mL penicillin/streptomycin) for 24 hours. Control conditions involved conditioning DC with complete medium only for 24 hours. Following conditioning, DC were washed, and used in a mixed-leucocyte reaction (MLR) with allogeneic T-cells.

2.3. Enrichment of Blood T-Cells. PBMC were suspended in MiniMACS buffer (PBS containing 0.5% BSA and 2 mM

EDTA) and T-cells were enriched by depletion of $CD14^+$, $CD19^+$ and $HLA\text{-}DR^+$ cells with immunomagnetic beads (Miltenyi Biotech, Bisley, UK) following manufacturer's instructions.

2.4. T-Cell Proliferation Assay. Carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen Ltd, UK) labelled T-cells (4×10^5/well) were incubated for 5 days in U-bottomed 96 well microtitre plates with enriched, previously conditioned, allogeneic DC at 0%, 1%, 2%, or 3% in a mixed leukocyte reaction (MLR). Cells were recovered and $CFSE^{lo}$ proliferating cells identified and quantified by flow cytometry.

2.5. Antibody Labelling. Monoclonal antibodies with the following specificities and conjugations were used: CLA-FITC (HECA-452), $\beta 7$ integrin-PE (FIB504), IL-12 (p40/p70)-PE (C11.5), IL-17A-PE (SCPL1362), CD3-PerCPCy5.5 (SK7), CD3-PeCy5 (UCHT1), IL-10-APC (JES3-19F1), IFNγ (25723.11), CLA-biotin (HECA-452) and Strepavidin-APC were purchased from BD Biosciences (Oxford, UK); CCR9 (either—FITC or—APC) (112509), CCR7-PE (150503), CCR10-APC (314315), CCR4-APC (205410) and TGFβ (IC388P) were purchased from R&D Systems (Abingdon, UK). Appropriate isotype-matched control antibodies were purchased from the same manufacturers. After the staining, cells were fixed with 1% paraformaldehyde in 0.85% saline and stored at 4°C prior to acquisition on the flow cytometer, within 48 hours.

2.6. Flow Cytometry and Data Analysis. Data were acquired on a FACSCanto II cytometer (BD Biosciences) and analysed using WinList 5.0 software (Verity, ME, US). Proportions of positive cells were measured by subtracting the appropriate isotype-matched control staining from test histogram using superenhanced D_{max} (SED) normalised subtraction.

2.7. Cytokine Analysis. The intracellular cytokine production by stimulated T-cells post-MLR was measured using superenhanced D_{max} (SED) normalised subtraction upon data analysis following incubation +/− monensin, T-cell permeabilisation, antibody labelling and flow cytometry.

2.8. Statistical Analyses. Data are presented as mean and standard errors. Two-way repeated measures ANOVA, and two-tailed paired t-tests were applied as stated in the figure legends. In the case of multiple comparisons, subsequent *ad-hoc* Bonferroni correction was applied. $P < 0.05$ was considered significant.

3. Results

3.1. Characteristics of Human DC Function in UC

3.1.1. Reduced T-Cell Stimulatory Capacity of DC in UC. We analysed DC stimulation of T-cells in a 5-day mixed leucocyte reaction (MLR). T-cells from the same donor (a separate, healthy control) were stimulated by DC from healthy controls and UC patients, within the same experiments. DC stimulated a strong, dose-dependent proliferative response in both healthy controls and UC patients; dividing T-cells were identified as $CFSE^{lo}$ $CD3^+$ lymphocytes, by flow cytometry (Figure 1(a)). However, DC from UC patients (UC-DC) stimulated a significantly weaker proliferation of the same CFSE-labelled T-cells compared with DC from healthy controls (control DC; Figure 1(b)).

3.1.2. DC in UC Exhibit an Enhanced Ability to Imprint Skin-Homing Properties on Effector T-Cells. We have previously demonstrated T-cells within fresh PBMC expressed *either* gut-homing molecule $\beta 7$ or skin-homing molecule CLA; the majority expressed $\beta 7$ only. Freshly purified T-cells exhibited the same homing profile, prior to co-culture with allogeneic DC [37]. Post-culture, the expression of $\beta 7$ on dividing T-cells ($CFSE^{lo}$) was the default pathway; T-cells stimulated by both control and UC-DC maintained $\beta 7$ expression, as did unstimulated T-cells. In contrast, CLA expression was induced on dividing T-cells by both control and UC-DC so that substantial numbers of T-cells were identified as double positive for CLA and $\beta 7$ following stimulation (due to inherent high expression of $\beta 7$ in all conditions; Figure 2(a)). However, UC-DC exhibited an enhanced ability to prime skin-homing T-cells, significantly increasing the proportion of total CLA^+ T-cells (Figure 2(b)) and the proportion of T-cells expressing skin-homing molecule CCR4 (Figure 2(c)) within the stimulated population.

3.2. Effects of LcS Treatment on Dendritic Cell Function

3.2.1. LcS Restored T-Cell Stimulatory Capacity of Dendritic Cells in UC. Optimisation experiments on healthy control DC determined no significant differences between live or heat-killed (HK) LcS regarding ability to enhance DC activation/maturation markers CD80 and CD83; both live and HK LcS significantly enhanced CD80 and CD83 expression (Figure 3(a)). Therefore HK LcS was used for all further experiments.

We analysed DC stimulation of T-cells in a 5-day mixed leucocyte reaction (MLR) following DC conditioning with complete medium only, or varying concentrations of HK LcS (1×10^5, 1×10^6, or 1×10^7 CFU/mL). A significant, dose-dependent increase in DC stimulatory capacity was observed upon LcS conditioning of both control- and UC-DC (Figure 3(b)). Following LcS conditioning, UC-DC levels of stimulation were restored to "normal" levels, similar to that of control DC (Figure 3(c)).

3.2.2. LcS Conditioned DC to Imprint Skin-Homing Properties on T-Cells in Healthy Controls but Not UC Patients. LcS conditioning of DC had differential effects in healthy controls compared with UC, on DC ability to imprint homing properties on stimulated T-cells. In healthy controls, LcS conditioning enhanced DC ability to induce a skin-homing profile on T-cells, significantly increasing the proportion of stimulated T-cells expressing CLA, in a dose-dependent fashion (Figures 4(a) and 4(b)). However, in UC, CLA

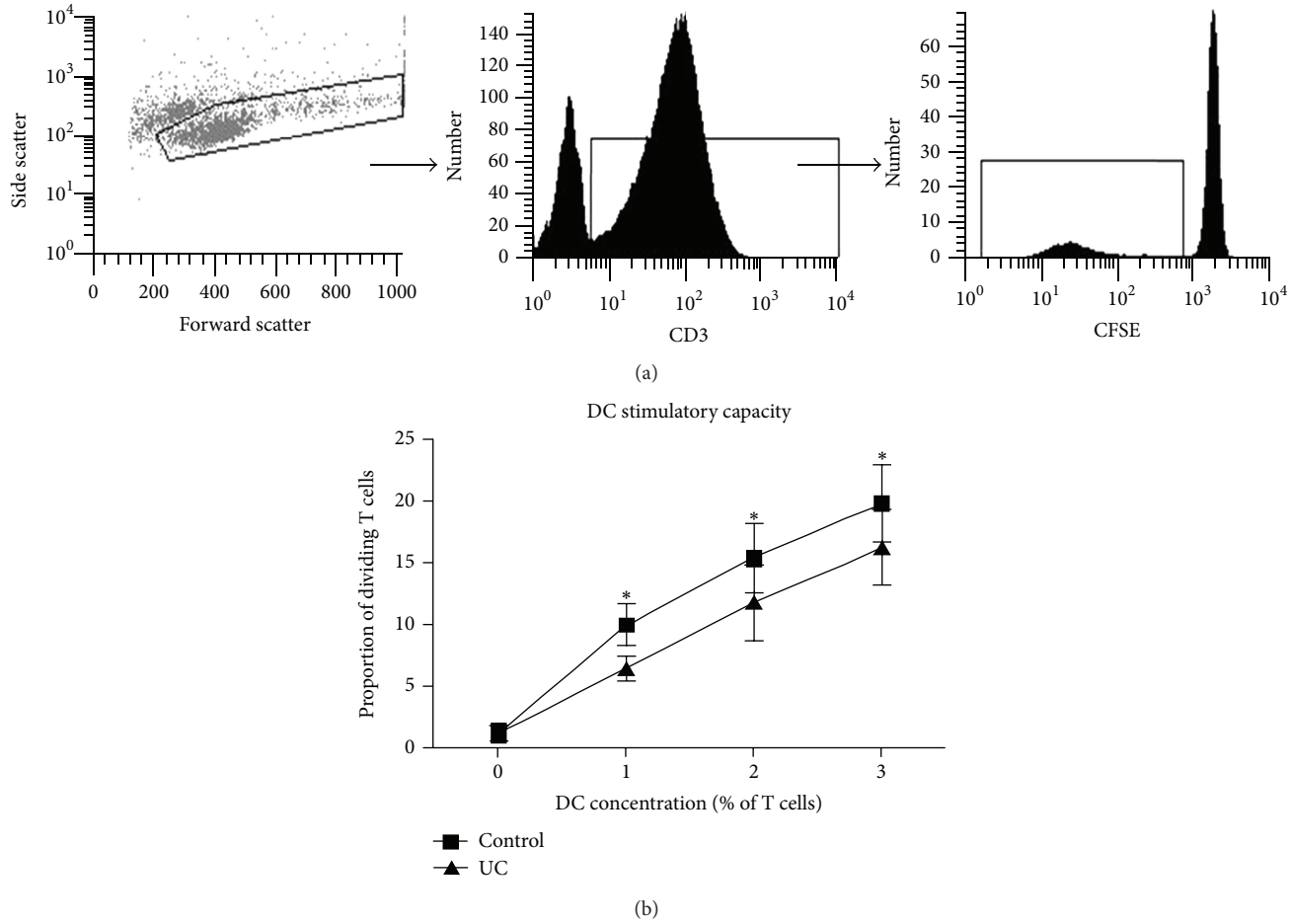


Figure 1: Restricted DC stimulatory capacity in UC. (a) Identification of dividing T-cells following mixed leucocyte reaction (MLR) according to flow cytometry forward and side scatter dot plot, and subsequent CD3 and CFSE histograms, respectively. (b) Dose response T-cell proliferation following MLR. Results are displayed as mean ± SEM ($n = 6$). Base-level proliferation is shown as proportion of dividing T-cells with no DC (0%). After paired two-way ANOVA analysis (corrected with Bonferroni correction for multiple comparisons), the DC concentration was statistically significant in both cases ($P < 0.01$) that is, a dose response occurred in both cases. UC-DC were less stimulatory than control DC ($P < 0.05$ at 1%, 2%, and 3% DC).

expression on T-cells was already enhanced (Figures 2(a) and 2(b)), and LcS conditioning had no further effects on DC ability to enhance CLA expression on T-cells (Figure 4(b)). LcS conditioning had no effect on DC ability to induce CCR4 expression in either healthy controls or UC patients (data not shown).

CLA expression on T-cells was enhanced upon stimulation by both untreated UC-DC and LcS-conditioned (control) DC. Induction of CLA on T-cells stimulated by LcS-conditioned DC from controls was in conjunction with gut-homing marker $\beta 7$. However, CLA induction by untreated UC-DC was on the $\beta 7$ negative fraction of T-cells (Figure 4(c)). Thus, the proportion of $CLA^+\beta 7^-$ T-cells within the total CLA^+ dividing T-cell pool was significantly greater upon stimulation with UC-DC, compared to LcS (1×10^7 CFU/mL) conditioned (control) DC (Figure 4(d)).

3.2.3. LcS Conditioned DC to Induce TGFβ Production by T-Cells in Healthy Controls but Not UC Patients. LcS conditioning of DC also had differential effects on DC ability to induce cytokine production by stimulated T-cells, in controls compared with UC patients. Although there were differences within individual experiments between the ability of control DC and UC-DC (both untreated) to induce cytokine production by T-cells (IL-10, TGFβ, IFNγ and IL-17A were measured), overall there were no significant differences (Figure 5(a)). However, TGFβ production by T-cells was significantly increased, in a dose-dependent manner, when DC were conditioned with LcS in healthy controls, but not in ulcerative colitis (Figure 5(b)).

4. Discussion

We demonstrate for the first time, that human circulating DC from UC patients exhibit a restricted stimulatory capacity for allogeneic T-cells, and these DC induce a specific skin-homing profile on stimulated T-cells that DC from healthy controls do not. Our data support studies demonstrating dysregulated DC function in IBD [11, 15, 16], and furthermore, demonstrate systemic immune dysregulation in IBD patients

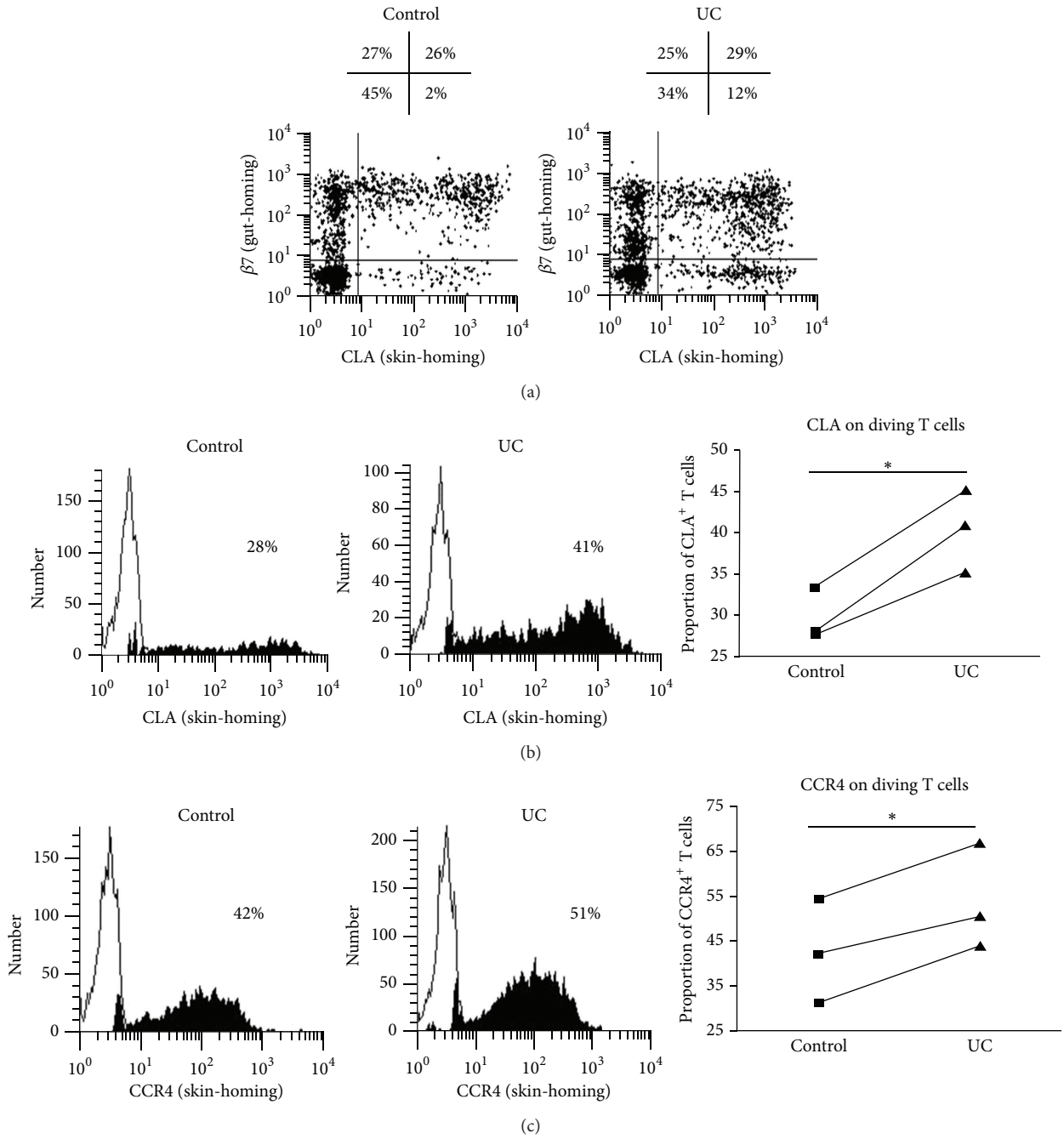
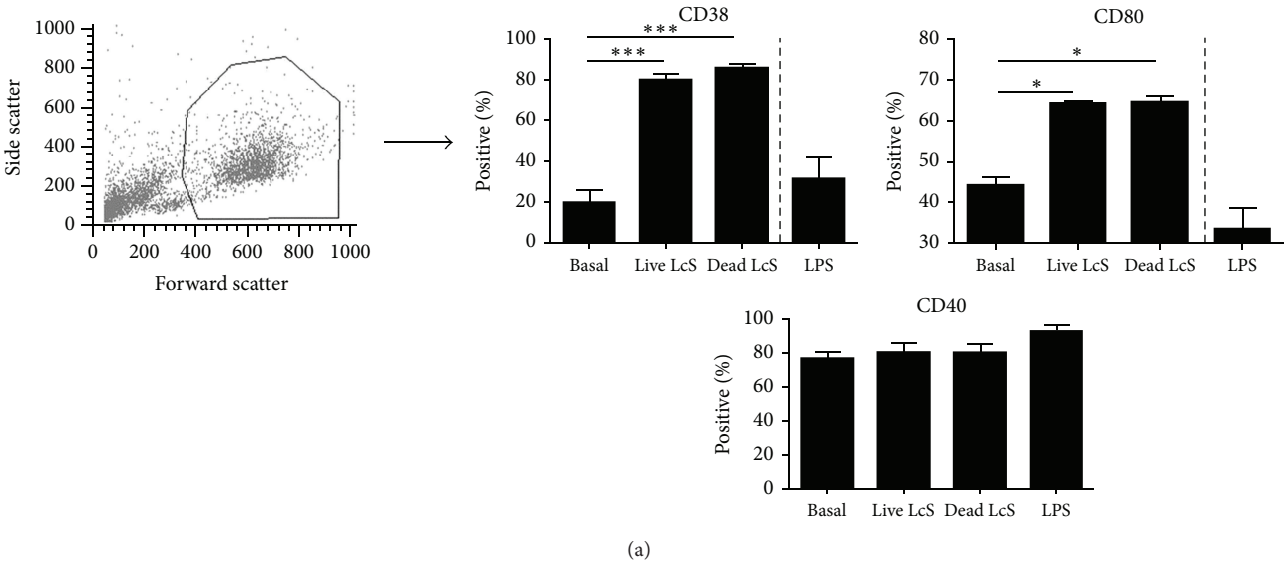
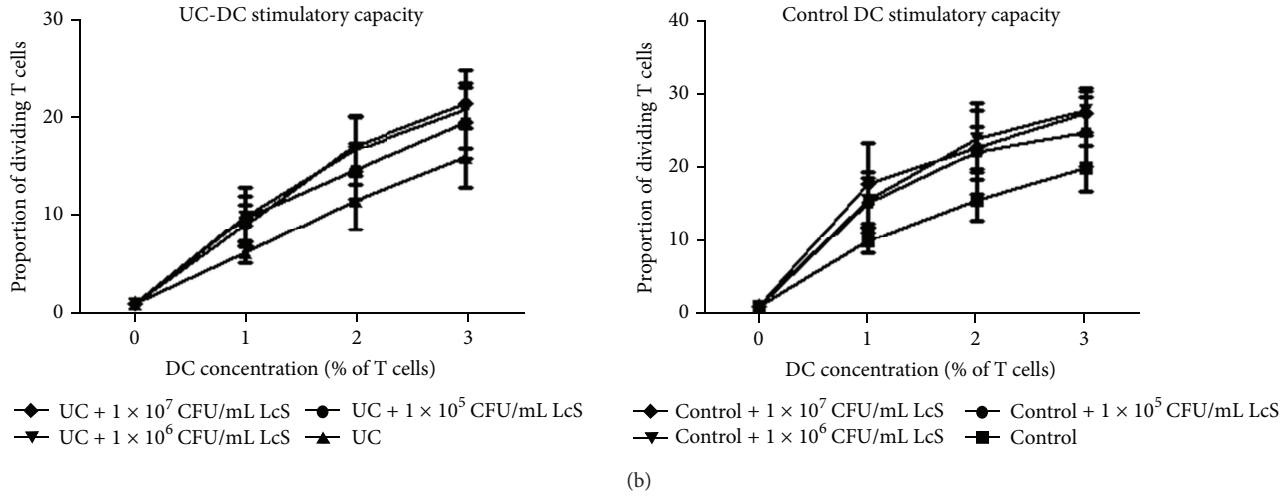


Figure 2: UC-DC enhanced expression of skin-homing molecules on stimulated T-cells. (a) CLA/$\beta7$ dot plots of dividing T-cells, following stimulation by 3% control or UC-DC. Numbers over each dot plot represent proportion of dividing T-cells expressing $\beta7$ only, $\beta7$ and CLA, CLA only or neither $\beta7$ or CLA. (b) Histograms of CLA expression by dividing T-cells, following stimulation by 3% control or UC-DC. On the right, summary graph of all experiments ($n = 3$). (c) Histograms of CCR4 expression by dividing T-cells, following stimulation by 3% control or UC-DC. On the right, summary graph of all experiments ($n = 3$). Paired t-test was applied, P-value <0.05 was considered statistically significant ($^*P < 0.05$). All representative histograms/dot plots are from a single experiment representative of 3 independent experiments performed with similar results. Filled histograms represent positive staining, empty histograms represent background staining.

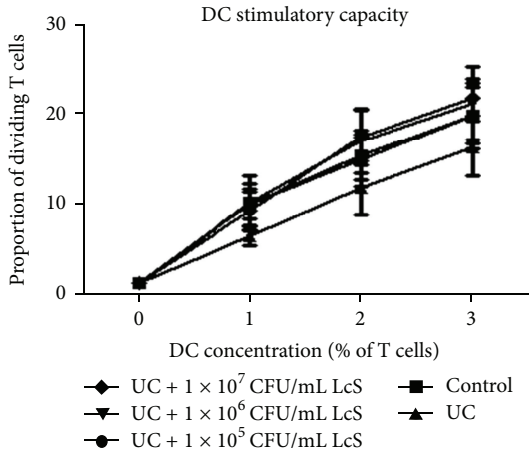
rather than at mucosal sites only. The occurrence of extra-intestinal manifestations (EIM) associated with IBD indicates IBD is indeed a systemic disease, and our data provide an explanation for the occurrence of EIM affecting the skin [17]. Conditioning UC-DC with probiotic strain LcS restored their stimulatory capacity, reflecting that of control DC. LcS had differential effects on DC in healthy controls and UC, on DC ability to imprint specific homing profiles on stimulated T-cells, and to induce cytokine production by T-cells. This is the first study, to our knowledge, to investigate the effects of probiotic bacteria on migratory properties of immune cells. Our data supports studies demonstrating



(a)

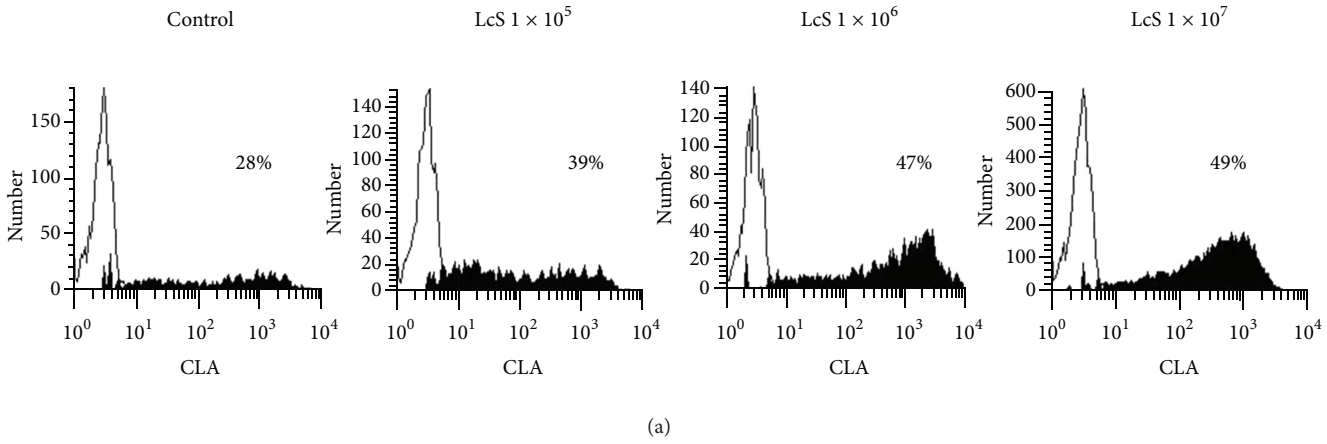


(b)

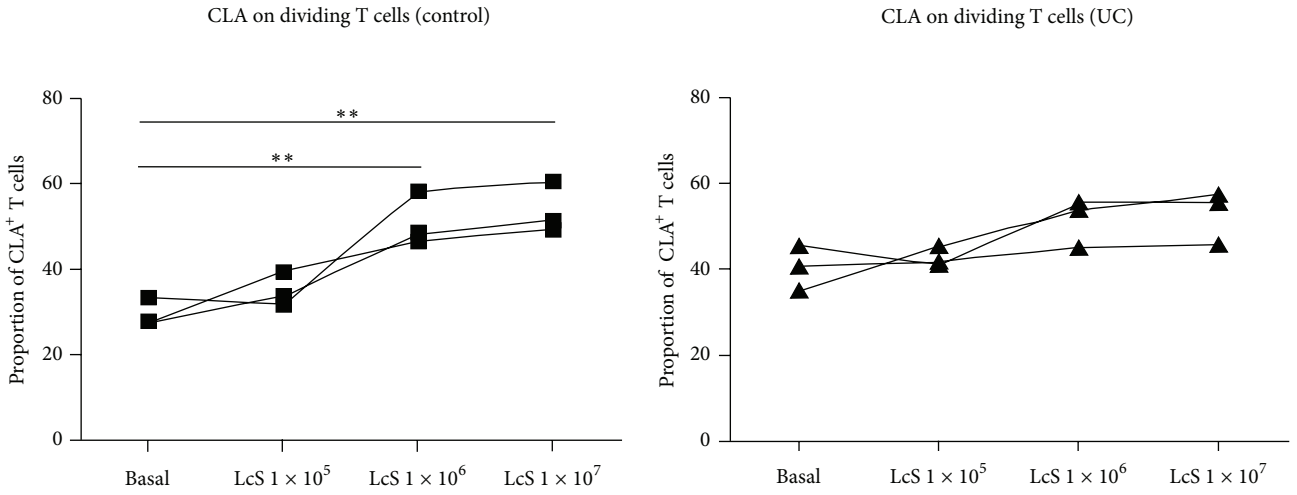


(c)

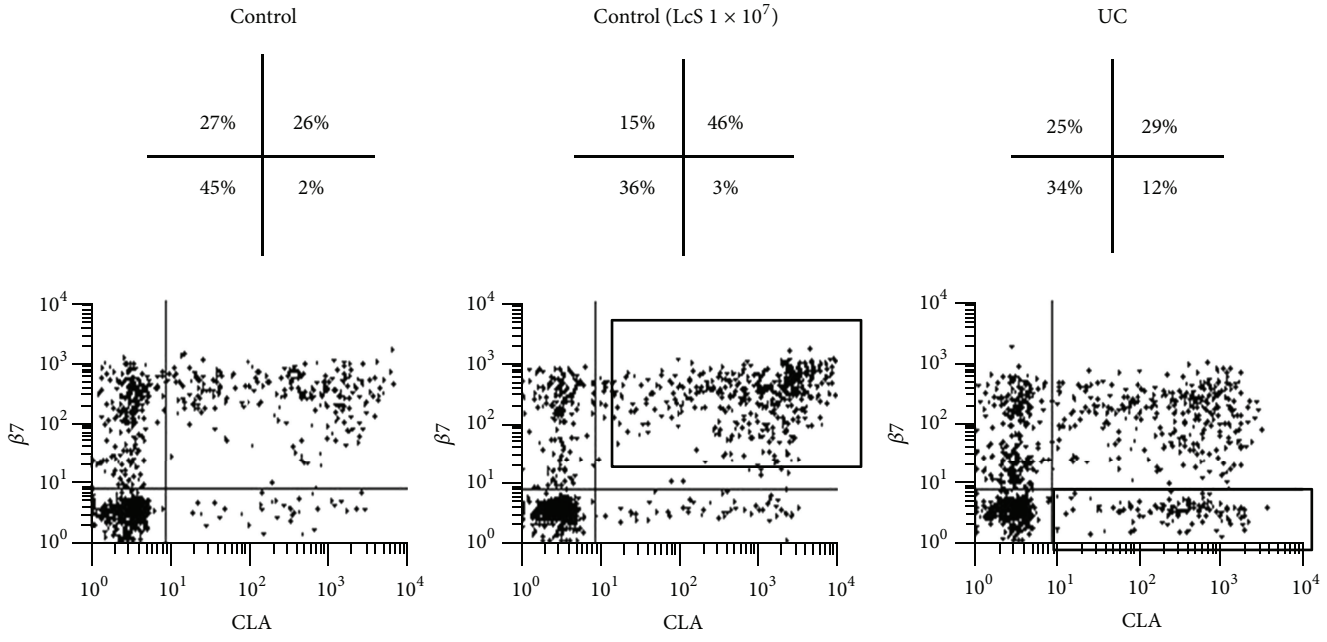
Figure 3: LcS restored "normal" DC stimulatory capacity in UC. (a) Identification of blood-enriched DC according to flow cytometry forward and side scatter plot and summary graphs representing mean ± SEM proportions of DC expressing CD40 and CD86 following conditioning with medium only, live LcS or dead LcS ($n = 3$). Separate experiments were carried out conditioning DC with LPS ($n = 3$). (b) Dose response T-cell proliferation following MLR with control- and UC-DC ($n = 6$). After paired two-way ANOVA analysis (corrected with Bonferroni correction for multiple comparisons), the DC concentration was statistically significant in all cases ($P < 0.01$). Control- and UC-DC stimulatory capacity was increased following LcS conditioning at LcS concentrations of 1×10^5 (control: $P < 0.05$ at 2%, UC: $P < 0.01$ at 1%, $P < 0.05$ at 2%, $P < 0.01$ at 3% DC), 1×10^6 (control: $P < 0.01$ at 2% and 3%, UC: $P < 0.05$ at 2%, $P < 0.01$ at 3% DC) and 1×10^7 (control: $P < 0.01$ at 1%, 2%, and 3%, UC: $P < 0.01$ at 2%, and 3%) CFU/mL. (c) There were no significant differences between the stimulatory capacity of control and LcS-conditioned UC-DC at any LcS concentration.



(a)



(b)



(c)

Figure 4: Continued.

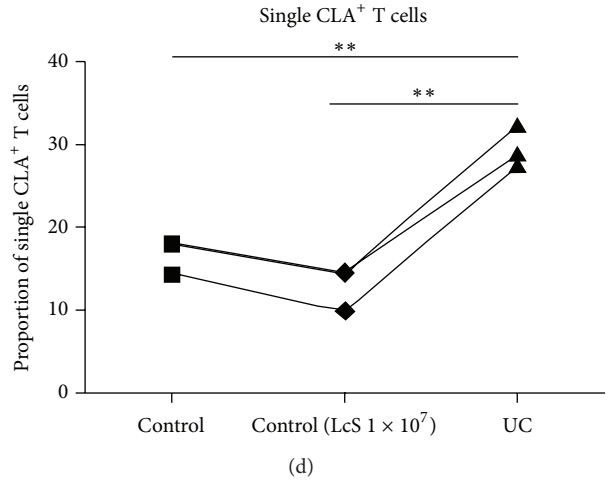


Figure 4: LcS conditioning of DC enhanced expression of CLA on stimulated T-cells in healthy controls but not UC. (a) Histograms of CLA expression by dividing T-cells, following stimulation by 3% DC from healthy control (no LcS) and after conditioning with $1 \times 10^5/1 \times 10^6/1 \times 10^7$ CFU/mL LcS. Example is from one experiment but representative of 3 independent experiments with similar results. (b) Summary graphs of all experiments, representing proportions of CLA^+ T-cells stimulated by 3% control and UC-DC conditioned with increasing doses of LcS ($n = 3$). One way-ANOVA was applied, P-value <0.05 was considered statistically significant ($^*P < 0.05$, $^{**}P < 0.01$). (c) CLA/β7 dot plots of dividing T-cells, following stimulation by 3% control DC, control DC + 1×10^7 LcS or UC-DC (no LcS). Example is from one experiment but representative of 3 independent experiments with similar results. Numbers over each dot plots represent proportion of dividing T-cells expressing β7 only, β7 and CLA, CLA only or neither β7 or CLA. (d) Summary graph of all experiments ($n = 3$) representing the proportion of dividing T-cells (stimulated by 3% DC in all cases) expressing CLA only (i.e., β7 negative), out of total CLA^+ dividing T-cells. Paired t-test was applied, P-value <0.05 was considered statistically significant ($^*P < 0.05$, $^{**}P < 0.01$).

multifunctional immunoregulatory activities of LcS, depending on the responding cell types and the local microenvironment [38].

LcS conditioned control DC, but not UC-DC, to imprint skin-homing molecule CLA on stimulated T-cells. However, unlike the skin-homing profile induced on T-cells by UC-DC, CLA expression induced via LcS conditioning was in conjunction with gut-homing molecule β7, suggesting induction of a multi-homing profile. The differential effects of LcS on control and UC-DC were further demonstrated by the induction of TGFβ production by T-cells stimulated with LcS-conditioned DC in controls but not UC patients. These data suggest effects of LcS exerted on human DC are flexible, depending on the responding cell types and the local cytokine environment. The restoration of UC-DC stimulatory capacity by LcS suggests LcS may partly contribute to restoration/maintenance of homeostasis.

LcS may also confer homeostatic properties at intestinal sites (e.g., via oral administration) which could be beneficial in IBD; gut DC play a central role in immune homeostasis in the gut [39] and exhibit tolerogenic properties [15]. Alterations occur in gut DC in IBD [15, 16], leading to loss of tolerance in the gut and dysregulated immune responses to the colonic microbiota, a major contributing factor in the onset of IBD [11]. Restoration of homeostatic properties of gut DC by LcS at *intestinal* sites may account for the reported efficacy of LcS in UC [32]. However, the local microenvironment and responding cell types differ dramatically in the circulation and the gut for example, gut DC are conditioned by intestinal epithelial cells and epithelial cell-derived products to adopt their tolerogenic function [40–42]. Future studies will determine *in vitro* effects of LcS on gut DC and also on epithelial cell conditioning of gut DC.

Although the definition of probiotics involves *live* microorganisms (which, when administered in adequate amounts confers health benefit on the host) [2], our data demonstrates immunomodulation by *heat-killed* bacteria; furthermore, we demonstrated no significant differences between live and HK LcS regarding their ability to enhance activation marker expression on blood-enriched DC from healthy controls. These data support studies demonstrating immunomodulation by probiotic bacterial products, including the ability of probiotic bacterial DNA to induce regulatory IL-10 production by human peripheral blood mononuclear cells [43] and dendritic cells [44], and the ability of sonicated probiotic bacteria to induce marked anti-inflammatory effects on blood and intestinal DC. Furthermore, our recent studies have demonstrated an immunomodulatory peptide secreted by *Lactobacillus plantarum*, mediates some of the molecular dialogue between intestinal bacteria and DC, inducing immunoregulatory effects in both blood and intestinal DC *in vitro* [45].

IBD is associated with a variety of EIM, with up to a third of patients developing cutaneous manifestations including erythema nodosum (EN) and pyoderma gangrenosum (PG) [17]. The causes of EIM of IBD are poorly understood, but it has been suggested that compartmentalisation of inflammatory processes to different organs (e.g., intestine, skin, liver) may be linked to homing and trafficking of immune cells. For example, CCL25, the ligand for gut-homing receptor CCR9, is expressed on epithelium in both the liver and the small intestine [18]. Dysregulation of lymphocyte trafficking plays

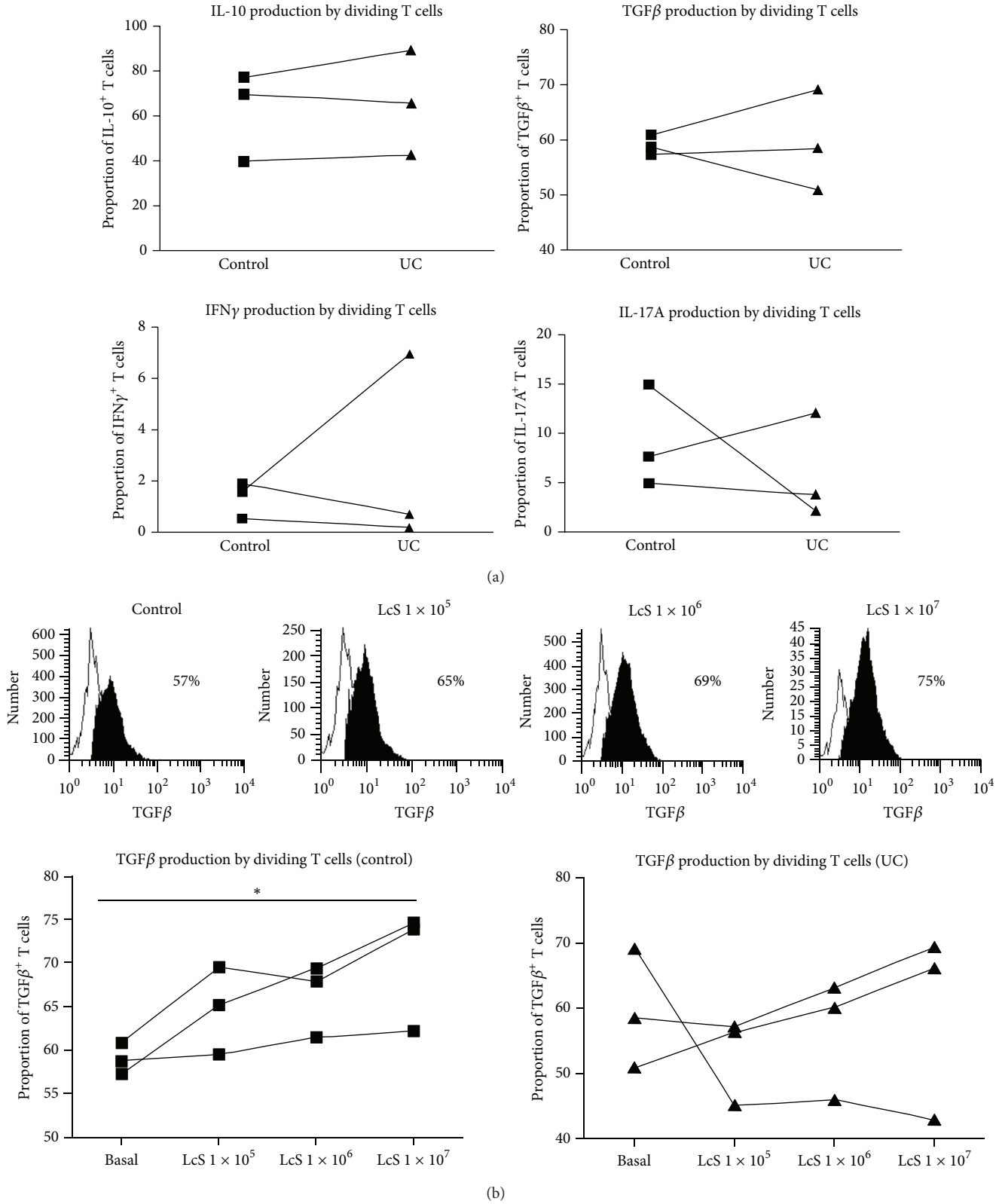


Figure 5: Intracellular cytokine production by stimulated T-cells. (a) Summary graphs of all experiments representing proportions of T-cells stimulated by 3% control and UC-DC producing TGFβ, IL-10, IFNγ and IL-17 ($n = 3$). Paired t-tests were applied, P-value <0.05 was considered statistically significant. (b) Histograms of TGFβ expression by dividing T-cells, following stimulation by 3% control (no LcS) DC or control DC + $1 \times 10^5/1 \times 10^6$ or 1×10^7 CFU/mL LcS. Example is from one experiment but representative of 3 independent experiments with similar results. Below, summary graphs of all experiments ($n = 3$) representing the proportion of dividing T-cells (stimulated by 3% DC in all cases) producing TGFβ. DC were from healthy controls (left graph) or UC patients (right graph). One way-ANOVA was applied, P-value <0.05 was considered statistically significant ($^*P < 0.05$).

a key role in IBD pathogenesis [19–22, 46] and IBD therapeutics have previously demonstrated efficacy by abrogating trafficking of effector cells to intestinal sites [47–51]. However, we demonstrate in this study that skin-homing markers CLA and CCR4 are aberrantly expressed on $\beta 7^-$ T-cells stimulated by UC-DC, providing an explanation for the occurrence of EIM affecting the skin, and supporting previous studies demonstrating conditioning DC with supernatants from culture of colonic biopsies from UC patients enables them to imprint a skin-homing phenotype on stimulated T-cells [52]. Blocking trafficking of effector cells to *cutaneous sites* in patients with EIM of IBD may also be of therapeutic benefit.

Although there were no significant effects of LcS on DC ability to induce T-cell cytokine production in UC overall, effects of LcS were variable between individual experiments, depending on whether production of particular cytokines were increased or decreased compared to T-cells stimulated by control DC (data not shown). These data also suggest restoration of a "normal" phenotype and support a multifunctional immunoregulatory role for LcS, returning dysregulated immune functions to the original normal state when the host becomes either immunocompromised or excessively activated [38]. Indeed, LcS can have either pro- or anti-inflammatory effects in human intervention studies [32, 53, 54] and *in vitro* studies [55–57] depending on the context.

In summary, our data demonstrate systemic alterations in immune cells in UC, specifically a dysregulated DC function. Our data provides an explanation for the occurrence of EIM of the skin in UC patients, and suggests that the probiotic strain LcS has multifunctional immunoregulatory activities on DC, depending on the disease state and the inflammatory environment. Our data supports studies demonstrating probiotic bacterial products, rather than live bacteria are capable of inducing immunoregulatory effects. The reported therapeutic effects of LcS and other probiotic *Lactobacilli* strains in UC [32, 58] may be partly due to promotion of homeostasis, restoring the dysregulated functions of immune cells.

Acknowledgments

This research was funded by Yakult Europe B.V. (Almere, The Netherlands) and the *L. casei* Shirota was provided by Yakult Honsha Co. Ltd. (Tokyo, Japan). Grant support: Yakult Europe B.V. (The Netherlands).

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 608187, 11 pages
http://dx.doi.org/10.1155/2013/608187

Review Article

The Role of IL-33 in Gut Mucosal Inflammation

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Received 4 January 2013; Accepted 9 May 2013

Academic Editor: Eduardo Arranz

Interleukin (IL)-33 is a recently identified cytokine belonging to the IL-1 family that is widely expressed throughout the body and has the ability to induce Th2 immune responses. In addition, IL-33 plays a key role in promoting host defenses against parasites through the expansion of a novel population of innate lymphoid cells. In recent years, a growing body of evidence has shown that the proinflammatory properties displayed by IL-33 are detrimental in several experimental models of inflammation; in others, however, IL-33 appears to have protective functions. In 2010, four different research groups consistently described the upregulation of IL-33 in patients with inflammatory bowel disease (IBD). Animal models of IBD were subsequently utilized in order to mechanistically determine the precise role of IL-33 in chronic intestinal inflammation, without, however, reaching conclusive evidence demonstrating whether IL-33 is pathogenic or protective. Indeed, data generated from these studies suggest that IL-33 may possess dichotomous functions, enhancing inflammatory responses on one hand and promoting epithelial integrity on the other. This review focuses on the available data regarding IL-33/ST2 in the physiological and inflammatory states of the gut in order to speculate on the possible roles of this novel IL-1 family member in intestinal inflammation.

1. Introduction

The intestinal epithelium is the largest surface area of the human body in direct contact with the external environment and exposed to a multitude of foreign microorganisms, macromolecules, and xenobiotics. As such, a fine regulation of gut mucosal immune functions is needed to develop a prompt and self-limiting inflammatory response against harmful pathogens but also to maintain normal gut homeostasis when no potential threat is sensed. Complex interactions between different cell types, effectors of both innate and adaptive immunity, regulate the inflammatory status within the intestinal mucosa. Pro- and anti-inflammatory cytokines represent key players in shaping this network and maintaining the communication among various cell types; balance among these mediators appears to be critical for gut immune homeostasis. In fact, a broad wealth of evidence demonstrates the importance of cytokine dysregulation in the onset of inflammatory conditions of the gastrointestinal tract. In particular, IBD, namely, Crohn's disease (CD) and ulcerative colitis (UC), is characterized by a significant dysregulation of cytokine production, with, in general, an overabundance of proinflammatory mediators [1]. For example, it has been shown that in the inflamed mucosa of IBD patients and colitis models, there is a perturbation of the balance between the proinflammatory cytokine, IL-1, and its naturally occurring antagonist, the IL-1 receptor antagonist (IL-1Ra), and that restoring this balance by exogenous administration of IL-1Ra ameliorates intestinal inflammation [2, 3]. As in the case of IL-1, other members of the IL-1 family, such as IL-18, have also been implicated in the initiation and perpetuation of chronic intestinal inflammation [4–6].

The IL-1 family of cytokines is constantly expanding, and, very recently, new members have been identified and studied, such as IL-1F11, IL-1F6/8/9, IL-1F7, and IL1F10, respectively known as IL-33, IL-36, IL-37, and IL-38 [7]. To date, of these novel members, IL-33 is the best characterized in terms of function and biological effects since its initial description in 2005 [8]. However, controversy still exists as to its precise role in intestinal disorders, particularly in the development of IBD. Thus, the aim of this review is to summarize what is already established regarding the role of IL-33 in the GI tract,

while providing insight into the potential role of this novel IL-1 family member in the pathogenesis of chronic intestinal inflammation.

2. IL-33: A Novel Member of the IL-1 Family

In 2003, a novel 30 kD protein, localized the nuclei of endothelial cells, was identified and shown to be highly expressed in high endothelial venules of tonsils, Peyer's patches, and lymph nodes [9]. The authors recognized, within the amino-terminal part of this molecule (aa 1–160), a novel homeodomain-like Helix-Turn-Helix (HTH) DNA-binding domain. As such, this protein was hypothesized to possess nuclear factor function, critical for the induction of a lymphatic endothelium phenotype, and was therefore coined "Nuclear Factor-High Endothelial Venules" (NF-HEV) [9]. Two years later, NF-HEV was identified as a novel member of the IL-1 family, shown to be the ligand for the former orphaned receptor, ST2, and renamed IL-1F11 or IL-33 [8]. In this first report, IL-33 was described as a potent enhancer of Th2 responses, inducing the production of IL-5 and IL-13. IL-33 was reported to be widely expressed in different cell types and within most organs throughout the body. In fact, IL-33 has been detected in cells of both hematopoietic origin, particularly in restricted populations of professional antigen presenting cells such as macrophages and dendritic cells, and in several different cell types of nonhematopoietic origin such as fibroblasts, adipocytes, smooth muscle cells, endothelial cells, bronchial and intestinal epithelial cells [8].

Initially, it was thought that IL-33 was synthesized as a full-length 30 kD protein (full-length IL-33, f-IL-33) and processed by caspase-1 upon inflammasome activation, resulting in an alleged 18 kD bioactive peptide in a similar fashion to the other major IL-1 family members, such as IL-1β and IL-18 [8]. However, further investigation by three independent research groups revealed that the inflammasome paradigm for IL-33 did not occur in the *in vivo* setting and, instead, demonstrated that f-IL-33 possessed full bioactivity, while proapoptotic caspase-3 and -7 processed f-IL-33 into less bioactive forms of 20–22 kD (cleaved IL-33, c-IL-33) [10–12]. More recently, f-IL-33 has been shown to serve as a substrate for neutrophil elastase and cathepsin G, resulting in 18–22 kD peptides with an increased bioactivity of tenfold, suggesting a possible extracellular mechanism to amplify the effects of IL-33 during inflammatory conditions [13]. To add further complexity to this scenario, an alternative splice variant of IL-33 (spIL-33) has been described that is 5 kD smaller than f-IL-33 and lacks the exons cleavable by caspases, but possesses similar bioactivity to f-IL-33 [14]. Figure 1 summarizes the current knowledge regarding the different IL-33 isoforms/splice variants and their bioactivity.

As previously mentioned, IL-33 exerts its biological effects through the binding of its receptor, ST2, also known as IL-1 receptor-like 1 (IL1RL1), belonging to the Toll-IL-1 Receptor (TIR) superfamily [8]. ST2 exists in two different splice variants, leading to the synthesis of proteins with opposite biological functions: ST2L, a transmembrane receptor that activates downstream signaling upon IL-33 recognition, and sST2, a soluble molecule that likely serves as a decoy receptor by binding IL-33 and decreasing its availability to ST2L, the IL-33 signaling receptor [15]. Similar to other TIR receptors, ST2L requires pairing to a coreceptor in order to initiate the downstream cell signaling cascade. As such, the IL-33 receptor complex consists of ST2L and the IL-1 receptor accessory protein (IL1RAcP), a TIR member also involved in IL-18 signaling [16]. ST2 and IL1RAcP interact through their TIR domain with MyD88, TRAF6, and IRAK1/4, eventually leading to the activation of transcription factors, such as NF-κB and AP-1, which promote the production of several proinflammatory mediators [8]. Another IL-1 family coreceptor member, that is, "single Ig IL-1R-related molecule" (SIGIRR) or Tir8, can also dimerize with ST2 and likely acts as a negative regulator of the IL-33/ST2 signaling pathway, ultimately reducing IL-33's biological effects [17]. To date, a very limited amount of information is available regarding the biologic and pathophysiologic relevance of IL-33 isoforms/splice variants, ST2 splice variants, and alternative ST2/SIGIRR signaling.

3. IL-33 Is a Key Player in Mucosal Immunity against Intestinal Parasites

Since its first description, IL-33 has been reported to be localized in barrier epithelia within organs/tissues in direct contact with the external environment, including the skin, airway, and gut epithelia, suggesting a possible role of this cytokine in early immune responses against invasive pathogens [8]. Moreover, several studies consistently show that normal mice injected with recombinant IL-33 develop a marked epithelial cell hyperplasia in the pulmonary and GI tracts, together with an eosinophilic and mononuclear infiltration into the lamina propria, specifically localized in these barrier organs/tissues [18, 19]. Interestingly, the production of a thick mucus layer is one effective mechanism aimed to enhance epithelial barrier function and infers protection of these mucosal organs. It is commonly thought that these specific effects on intestinal epithelial cells are mediated by the Th2 cytokine IL-13 [20, 21], which is abundantly overexpressed after IL-33 stimulation/administration [8]; however, the possibility that IL-33 *per se* promotes epithelial differentiation towards a secretory type it may not be ruled out. In fact, IL-33 is also a potent inducer of Th2 cytokines that are pivotal in mounting potent immune responses against helminthes and fungi; in fact, early papers exploring IL-33 function have pointed out its fundamental role in eliminating intestinal parasites. For example, *Trichuris muris*, a nematode capable of infesting the GI tract, induces the production of high levels of IL-33 from the infected ceca of experimental animals [18]. In this experimental setting, the administration of IL-33 was shown to boost parasite clearance from host mice. Even though a significant increase of NK cells was detected in mesenteric lymph nodes (MLNs) from SCID and wild-type animals treated with IL-33, parasite clearance appeared to be mediated through T- and B-cell activation, as SCID mice failed to eliminate *T. muris* upon IL-33 administration [18]. IL-33 appeared to have similar protective functions, enhancing

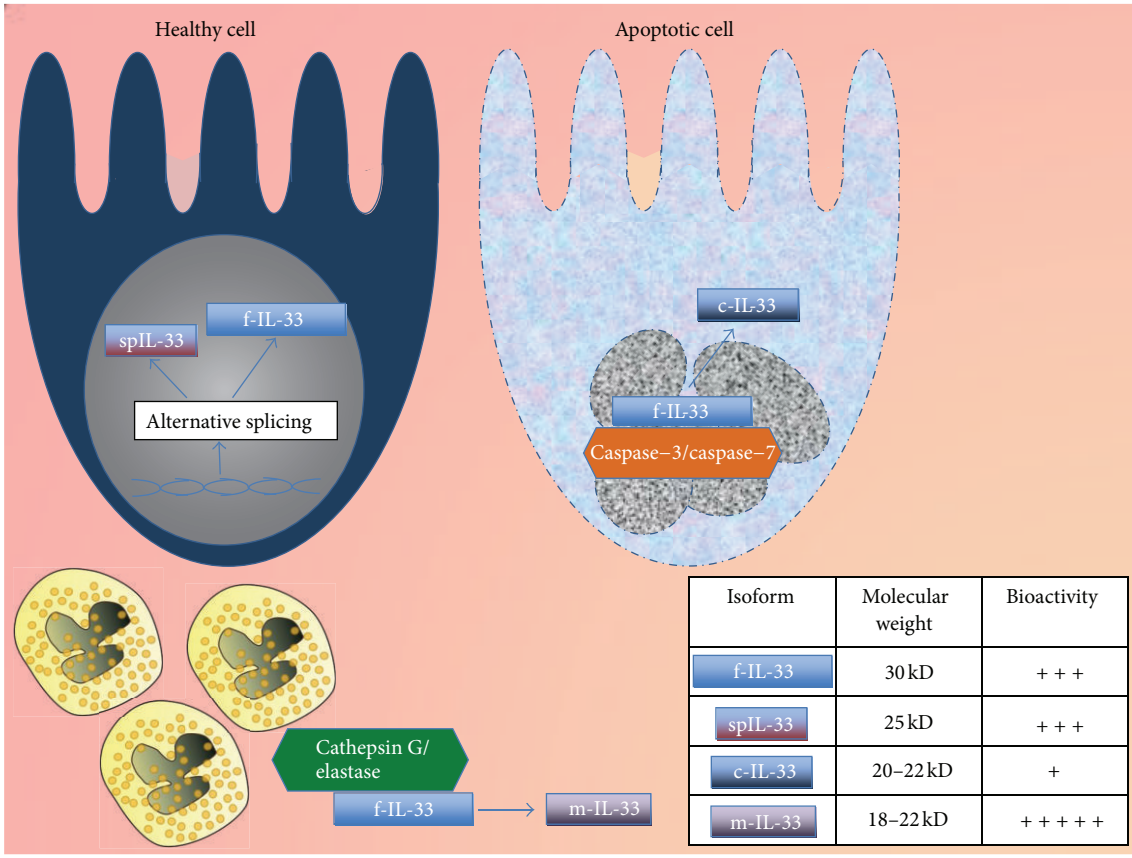

Figure 1: IL-33 isoforms and their associated bioactivity. IL-33 is synthesized as a 30 kD protein (full-length-IL-33, f-IL-33); however, alternative splicing can generate a 25 kD peptide (splice-IL-33, spIL-33), which possesses similar bioactivity to f-IL-33, but lacks caspase cleavage sites. During cellular apoptosis, f-IL-33 serves as a potential substrate for pro-apoptotic caspases (caspase-3 and caspase-7), generating smaller peptides of 20–22 kD with a marked reduction in bioactivity. Conversely, when secreted extracellularly, the proinflammatory activity of f-IL-33 may be potentiated in the context of a permissive, proinflammatory environment. In fact, Cathepsin G and elastase, released extracellularly by degranulating neutrophils, are able to cleave f-IL-33 into smaller isoforms (mature-IL-33, m-IL-33, 18–22 kD), which have been reported to display the greatest bioactivity.

host responses against other parasitic and bacterial threats, such as *Toxoplasma gondii* [22], *Pseudomonas aeruginosa* [23], and *Leptospira* [24] infections, in different organ systems.

More recently, a growing body of evidence has shown that IL-33's protective effects against parasites are also mediated by a newly identified innate immune cell population, uniquely coined "nuocytes," named after "nu," the 13th letter of the Greek alphabet, making reference to their ability to produce high levels of IL-13 [19]. In fact, these innate effector leukocytes display unique phenotypic characteristics and are not aligned with any other known mature leukocyte population. They express ICOS, CD45, ST2, and IL-17BR and respond to IL-33 and/or IL-25 stimulation with a significant increase in IL-13 expression [19]. In addition, the transcription factor, RORα, has been described to be necessary for their development [25]. Nuocytes appear to be early initiators of Th2 responses, and their activation is pivotal in eliciting worm clearance after *Nippostrongylus brasiliensis* infection, but a Th2 response is not required for nuocyte activity, as their development occurs in Th2 cytokine deficient mice and in RAG2 knockout mice [19, 26]. At the same time, similar IL-33/IL-25-responsive innate effector cell populations have been described by others. Moro et al. characterized a population, namely, natural helper cells (NHC), identified by the presence of cell surface ST2, c-Kit, Sca-1, and IL-7R, that reside in mesenteric adipose tissue and are organized in fat-associated lymphoid clusters [27]. Price et al. identified similar cells, but not expressing either Sca-1 or c-Kit, which were coined innate helper 2 (Ih2) cells and that are widely distributed in mouse MLN, spleen, liver, and bone marrow [28]. Together, these novel cell populations and others (e.g., multipotent progenitor type 2 ($MPP^{type\ 2}$) cells [29]) are termed innate lymphoid cells (ILCs) and share significant biologic similarities. As such, it may be hypothesized that ILCs represent different maturation steps or different differentiation phenotypes from the same hematopoietic lineage. Nonetheless, nuocytes, NHC, and Ih2 cells are induced by IL-33 and are pivotal in mounting effective immune responses against helminthes characterized

by the overproduction of IL-5 and IL-13 and the induction of histopathologic changes in the gut mucosa, including epithelial/goblet cell hyperplasia and eosinophilic infiltration. Whether or not these novel innate cell populations are the only responsible for the induction of these pathologic features is still debatable, as it has been clearly shown that IL-33 induces the production of chemokines for eosinophils [30] and may also directly affect epithelial cell biology. It seems reasonable that during parasitic infestation, in order to induce a prompt innate response, cells constituting intestinal barrier, such as epithelial cells and macrophages/dendritic cells, act as a major source of IL-33/IL-25; however, at present, no experimental data are available to confirm this hypothesis; novel experimental tools may provide important insights to this topic; in fact, mice genetically engineered to have the β-galactosidase-neomycin resistance fusion gene inserted in IL-33 intron 1, the IL-33-*LacZ* gene trap reporter mice, were recently described and used to specifically measure and localize IL-33 promoter activity within mouse body; indeed, these animals may be a valuable tool to identify the cellular sources of IL-33 during health and disease conditions, such as parasitic infestations. As a growing body of evidence confirms the importance of IL-33-induced ILCs in the protection against parasites, to date, the potential role of these novel cells in spontaneous inflammatory conditions has not been fully characterized in models of intestinal inflammation.

4. IL-33 and ST2 Are Dysregulated in IBD

IL-1 family members, coordinating early innate responses and later adaptive immune responses, have been shown to play an important role in the pathogenesis of chronic intestinal inflammation, characterizing IBD [31, 32]. As such, IL-33 appeared to be a promising candidate to be studied in the setting of human IBD. In fact, in 2010, four independent groups described the dysregulation of IL-33 expression in patients with UC and to a lesser extent, CD [33–36]. Consistently, all groups showed increased protein levels of IL-33 within the inflamed mucosa of IBD patients compared to healthy controls, particularly in UC [33–36]. Immunohistochemistry experiments revealed intense IL-33 staining in lamina propria inflammatory infiltrates, primarily localizing in cells that morphologically resemble macrophages and B cells/plasma cells [35]. Importantly, nonhematopoietic cell types also contribute to the augmented production of IL-33 during intestinal inflammation; in particular, intestinal epithelial cells [33, 35, 36] and myofibroblasts [34] display the highest levels of IL-33 during active IBD. IL-33 was also detected in other cell types within gut mucosa, confirming previous reports in other organ systems, with expression in fibroblasts, smooth muscle cells, endothelial cells [8, 37], and adipocytes [38]. IL-33 was also detectable in the sera of IBD patients, with concentrations significantly increased compared to healthy controls [33, 35]. Circulating IL-33 levels were also found to be markedly reduced upon anti-TNF administration (Infliximab/Remicade) and may have the potential to be used as a marker of disease activity and/or response to anti-TNF treatment [35]. Intriguingly, IL-33 isoforms vary according to the site wherein they are detected. In fact, f-IL-33 was the only form found to be present in both the cytoplasm and nuclei of IL-33-producing cells, such as intestinal epithelial cells, whereas evaluation of mucosal biopsies revealed the presence of both f-IL-33 and 20–22 kD cleaved forms; conversely, sera displayed exclusively the presence of the cleaved 20–22 kD forms [35]. Taken together, these data suggest the presence of extracellular proteases that have the ability to cleave IL-33, possibly modulating its bioactivity. As mentioned earlier, neutrophil elastase and cathepsin G are capable of cleaving f-IL-33, generating more potent forms [13]. As such, the inflammatory milieu characterizing IBD may have the potential to amplify IL-33's biological effects. On the other hand, similar to proapoptotic caspases, extracellular proteases may instead have the ability to inactivate f-IL-33, perhaps in an attempt to prevent possible harmful effects that may be triggered by high circulating levels of this cytokine. Indeed, further data are needed in order to clarify the significance of the circulating forms of IL-33 and the mechanism(s) leading to their generation.

Data regarding the analysis of IL-33 expression in human IBD were closely recapitulated in SAMP1/YitFc (SAMP) mice [35], a spontaneous model of chronic intestinal inflammation immunologically characterized by an early Th1 response and a later mixed Th1/Th2 phenotype [39], both significantly contributing to the extent of disease severity in these mice [40, 41]. In this study, SAMP mice were shown to display high IL-33 levels in the serum as well as the gut mucosa, consistently localized to intestinal epithelial cells and macrophages within the lamina propria. Interestingly, mucosal expression of IL-33 was found to positively correlate with the severity of SAMP enteritis [35].

A substantial alteration of ST2 expression as well was detected in the intestinal mucosa and sera from IBD patients. ST2 was abundantly expressed in the inflamed mucosa of IBD patients compared to healthy controls, and similarly, elevated circulating levels of sST2 were shown in UC and CD patients [33, 35], correlating with mucosal ST2 expression and both clinical and endoscopic disease activities [42]. Indeed, this interesting piece of data may suggest that serum IL-33/sST2 is produced within intestinal mucosa and directly reflects the severity of mucosal inflammation; as such, it would be worthwhile to investigate the role of circulating IL-33 and sST2 as markers of disease. Besides quantitative differences of ST2 expression, striking qualitative alterations were detected in the inflamed IBD mucosa versus healthy tissues. ST2 was constitutively expressed by intestinal epithelial cells during normal conditions; however, in chronically inflamed IBD mucosa, intestinal epithelial cells lose ST2 expression, which is redistributed to other inflammatory cell types. Specifically, intestinal epithelial cells of UC patients did not present ST2, whereas ST2 localized to lamina propria professional antigen presenting cells and T helper lymphocytes [35]. If a robust increase of ST2-positive cells within the lamina propria infiltrate is a common feature of different inflammatory conditions of the gut, the epithelial reduction/disappearance of ST2 appears to be specific for IBD. In fact, in nonspecific colitides, such as infectious colitis and diverticulitis, ST2 appears to be upregulated in both the epithelial and immune

compartments of gut mucosa [35]. Of note, epithelial dysregulation of ST2 refers to a marked decrease of ST2L, the IL-33 transmembrane receptor, but not of the sST2 protein [33, 35]. As such, this particular pattern of expression may suggest that during IBD, a severe impairment of IL-33 signaling within the epithelial layer occurs, whereas IL-33/ST2 engagement may be enhanced in intestinal immune cells. However, whether this alteration of epithelial ST2L is a feedback response to the chronic exposure of elevated IL-33 concentrations or an intrinsic epithelial defect characterizing IBD has yet to be determined.

Of note, during active IBD, an intense ST2 signal is detectable in perivisceral adipose tissue, where a rich infiltrate of ST2-positive immune cells is evident [35]. Consistent with recent data in the literature, this particular ST2-expressing cell population, dispersed within mesenteric fat, may represent the NHC population, recently described by Moro et al. [27], that are pivotal for the onset of immune responses against parasites, but whose possible role in idiopathic intestinal inflammation has not yet been explored.

Remarkably, as a further confirmation of the dysregulation of IL-33 and ST2 in human IBD, recent data, obtained in an Italian cohort of adult and pediatric UC and CD patients, demonstrate that specific IL-33 and ST2 gene polymorphisms confer an increased risk of developing IBD (both UC and CD), suggesting the involvement of the IL-33/ST2 axis in the onset of chronic intestinal inflammation [43].

5. The IL-33/ST2 Axis Exerts Dichotomous Functions during Idiopathic Intestinal Inflammation

Despite robust data describing the changes in patterns of the expression of IL-33 and ST2, current data regarding the role of this novel cytokine/receptor pair in the onset of IBD is conflicting and scarce. In fact, while some studies suggest a proinflammatory function, others indicate a protective, anti-inflammatory role.

Since its first description as a cytokine, IL-33 has been shown to possess potent proinflammatory activity, inducing Th2 cytokine production and promoting Th2 immunity. Thus, IL-33 was initially identified as a possible target for dampening inflammation in several animal models of inflammatory diseases, such as airway inflammation and arthritis (i.e., ovalbumin challenge-induced airways inflammation and collagen-induced arthritis) [44–46] since it was well established that IL-33 had the ability to promote inflammation through both the recruitment [47] and activation [48] of immune cells to the site of inflammation, as demonstrated by data obtained in a Th2 cell adoptive transfer model and *in vitro*. Consequently, mice overexpressing IL-33 were reported to display spontaneous airway and lung inflammation [49], whereas blocking the IL-33/ST2 axis decreased inflammation in an experimental murine model of asthma [44]. Similarly, antagonizing IL-33's biological effects, using anti-ST2 blocking antibodies, was effective in ameliorating joint inflammation in a murine model of rheumatoid arthritis [46]; in fact, the administration of IL-33 to cultures of immune cells isolated from murine inflamed joints led to a dramatic increase in IL-5, IL-6, and IL-17 production [45, 46]. These trends were also observed in a chronic model of intestinal inflammation [35]. Unfractionated MLN cells, collected from inflamed SAMP mice, secrete high levels of the aforementioned cytokines when cultured in the presence of IL-33 [35]. Of note, both IL-5 and IL-6 have been shown to play a pathogenic role in SAMP ileitis, as demonstrated by the amelioration of intestinal disease following either anti-IL-5 or anti-IL-6 treatment [50, 51], whereas IL-17 has been extensively characterized as a key cytokine in many immune-mediated diseases, including IBD [52–54]. Along these same lines, unpublished data generated in our laboratory showed that blockade of the IL-33/ST2 axis significantly reduces intestinal inflammation in SAMP mice [55–57]. Mechanistically, this effect appeared to be associated with a decrease of lamina propria eosinophil infiltration, by downregulating IL-5 and eotaxin-1 and eotaxin-2 [57], and by reducing the percentages of IL-17 producing macrophages [55], a population that has previously been described in intestinal and airway inflammatory conditions [58, 59]. However, a full phenotypic and functional characterization of this nonclassical cell population has not yet been performed. In addition, IL-33-induced inflammation may play a role in the development of intestinal fibrosis, as SAMP mice treated with anti-ST2 blocking antibodies show decreased collagen deposition within the intestinal wall, together with a reduced production of pro-fibrotic molecules, such as transforming growth factor (TGF)-β, connective tissue growth factor (CTGF), collagen-1, insulin growth factor (IGF)-1, and matrix metalloproteinase (MMP)-9 [56]. Consistently, healthy AKR mice treated for one week with intraperitoneal injections of recombinant IL-33 developed a marked thickening of muscularis layer of the intestinal wall, which was accompanied by increased expression of collagen-1, collagen-3, IGF-1, and CTGF [56]. These data, along with the observation that *in vitro* IL-33 stimulation of human subepithelial myofibroblasts (SEMF) induces the expression of profibrogenic genes such as *col1a1*, *col3a1*, *ctgf*, and *tgfb1* [56], suggest that IL-33 may also play an important role in promoting inflammation-associated gut fibrosis, as reviewed by Lopetuso et al. [60].

Additional data, primarily obtained from chemically induced models of intestinal inflammation, provide further insight into the role of IL-33 and ST2 in gut inflammatory conditions. Oboki et al. induced colitis in IL-33 knockout (KO) mice and wild-type littermates using dextran sodium sulfate (DSS) administration [61]. The colonic inflammation caused by DSS is primarily initiated by disruption of the epithelial barrier, which results in bacterial translocation into the underlying lamina propria. In this model, the resulting inflammation is mediated by the activation of innate immune responses and occurs in a T-cell-independent manner [62]. During the acute phase of this experimental model, IL-33 KO mice presented with reduced histologic inflammatory scores, with a significant reduction of granulocyte infiltration when compared to wild-type mice [61]. Other independent groups replicated similar results, using the same acute animal model [63–65]; in addition, Sedhom et al. confirmed the pathogenic role of IL-33 also in a different model of intestinal

inflammation, the trinitrobenzene-sulfonic-acid- (TNBS-) induced colitis, which is obtained throughout the chemical haptenization of protein expressed within the gut wall [64]. Interestingly, Sedhom et al. demonstrated on both DSS- and TNBS-induced colitis that, during the onset of intestinal inflammation, the IL-33/ST2 axis activation was able to affect the non-haematopoietic component of the inflammatory response, resulting in a significant impairment of the epithelial barrier function [64]. Conversely, results obtained by Oboki et al., during the recovery phase of the DSS-induced colitis in IL-33 KO mice, suggest that IL-33 might have different roles during different phases of the inflammatory process; in fact, weight recovery was markedly delayed in IL-33 KO mice, with a slight increase in mortality rate [61]. These results suggest that IL-33 is a critical amplifier of innate immune responses within the gut mucosa, whereas its role in the maintenance of chronic inflammation is less clear. Alternatively, IL-33 may possess an important functional role in enhancing innate immune responses related to bacterial clearance or in promoting mucosal wound healing.

Despite its well-known proinflammatory properties, IL-33 has also shown protective functions in different diseases; in fact, early reports demonstrated that IL-33 exerts a cardioprotective effect, reducing overload-induced cardiomyocyte hypertrophy [66]; on the same line, Miller et al. showed that IL-33 reduced the development of atherosclerosis [37] and the inflammation within the adipose tissue of obese mice [67], confirming the beneficial effect of IL-33 on the cardiovascular system. In addition, IL-33 appeared to have some protective role on various inflammatory conditions as well. For example, IL-33 appears to reduce inflammation in Con-A hepatitis [68], in central nervous system demyelinating disorders [69], and in pancreatitis [70]. Moreover, the ability of IL-33 to recruit neutrophils to the site of inflammation has been shown to reduce the consequences of severe septic events [71], whereas the IL-33-mediated expansion of IL-4-producing basophils, upon high-dose immunoglobulin administration, has been shown to induce profound immunoregulatory effects [72]. The dichotomous nature of IL-33 has also led to the generation of conflicting data in the setting of intestinal inflammation. In fact, data generated on the spontaneous enteritis characterizing SAMP mice suggests a frank pathogenic role, while IL-33 KO mice undergoing DSS colitis develop a mixed response. In addition, chronic DSS colitis appears to be less severe after IL-33 administration [73]. In these studies, Groβ et al. induced both acute and chronic DSS colitis in balb/c mice and administered IL-33 to experimental animals using different protocols. When IL-33 was injected during the first cycle of DSS, colonic inflammation was more severe, with a dramatic increase in neutrophil infiltration; conversely, treating animals during the recovery phases of both acute and chronic DSS colitis decreased inflammatory scores and improved epithelial regeneration [73]. A different group reported partially overlapping results using a similar acute DSS protocol. Utilizing the acute DSS colitis model, Imaeda et al. administered IL-33 every 48 hours. In these studies, the authors reported increased inflammatory scores in IL-33-treated balb/c mice; however, when evaluating the epithelial layer, complete reversion of the goblet cell depletion characteristic of DSS colitis was observed [63]. This effect appeared to be mediated by the suppression of Notch ligand expression by SEMFs. In fact, the Notch pathway is a key regulator of epithelial cell differentiation, leading towards an absorptive phenotype [74]. As such, IL-33-mediated inhibition of the Notch pathway resulted in the maturation of epithelial cells towards a goblet cell phenotype, likely representing a protective response against harmful conditions [63]. Interestingly and along similar lines, IL-33 expression has been reported in SEMFs underlying ulcerated epithelia in UC and gastric ulcers [34, 75]. In addition, recent data suggest that IL-33 stimulation may increase gastric epithelial proliferation [76], whereas dramatic changes in the pattern of IL-33 expression in endothelial cells have been described during angiogenesis [77]. Overall, these data together strongly suggest that the IL-33/ST2 axis may be implicated in the maintenance of intestinal barrier function, wherein perturbations may likely play an important role in the development of chronic inflammatory conditions of the gut. As such, we can speculate that IL-33 may enhance bacterial clearance by inducing early granulocyte infiltration, promoting epithelial differentiation towards a mucus-secreting phenotype, and by facilitating wound healing. The redistribution/loss of ST2L within the epithelial compartment described during UC [33, 35] is consistent with the goblet cell depletion characterizing this particular disease and can also account for a defective wound healing process that can contribute to the chronicity of the inflammatory process.

Data generated by Duan et al. using the Th1-driven TNBS-induced colitis, opposing to what was shown by Sedhom et al., suggested more immune-mediated/immunomodulatory properties of IL-33 [78]. Using this model, mice developed less severe colitis following intraperitoneal injections of IL-33, whereas anti-IL-33 antibody administration did not significantly affect intestinal inflammation. The anti-inflammatory effects of IL-33 appeared to be mediated by a decreased production of the prototypic Th1 cytokine, IFNγ, while the Th2 cytokines, IL-5 and IL-13, were found to be increased. The authors conclude that IL-33 has the ability to initiate Th1 to Th2 skewing. In addition, the authors also infer that IL-33 has the ability to promote tolerogenic dendritic cell development, which ultimately results in the expansion of the T regulatory cell population [78]. Thus, during Th1-driven inflammation, IL-33 may have the capability to modulate gut mucosal immune responses to a more Th2-driven phenotype and promote the expansion of regulatory cell types. The different functions of the IL-33/ST2 axis during intestinal inflammation in SAMP spontaneous enteritis and in the chemically induced DSS- and TNBS-induced colitis models are recapitulated in Figure 2.

6. IL-33 As an Alarmin: A Possible Unifying Solution

Indeed, the IL-33/ST2 axis appears to be widely represented throughout the whole body, having different, and sometimes opposing, functions. The resulting balance between the differential effects appears to be straightforward in certain

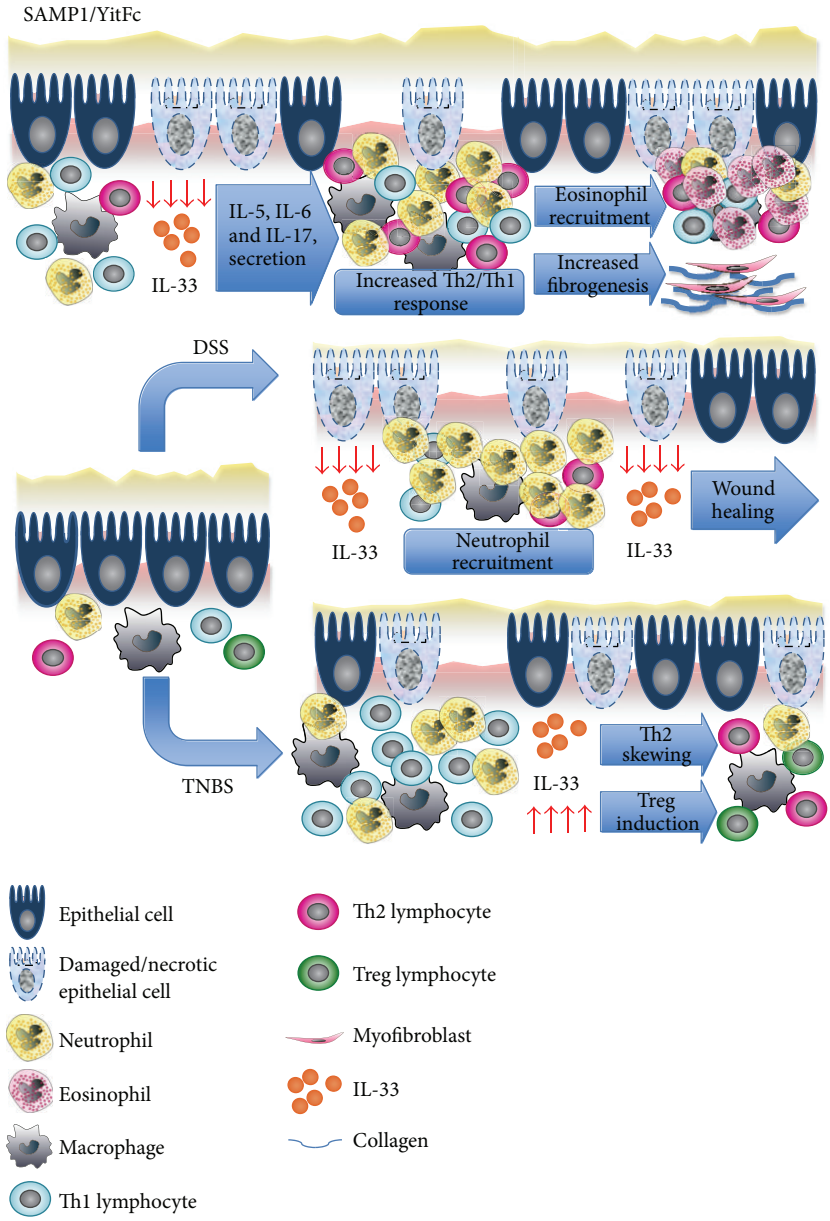

Figure 2: Role of IL-33 in murine models of intestinal inflammation. Experimental results obtained from SAMP mice that develop spontaneous Th1/Th2-driven enteritis suggest a pathogenic role for IL-33 at the onset of intestinal inflammation in this specific model. In fact, epithelial-derived IL-33 promotes the release of proinflammatory cytokines from LP immune cells, enhancing both Th1 and Th2 responses. Moreover, IL-33 induces the production of eotaxin-1 and eotaxin-2, which leads to eosinophil chemotaxis to the inflamed gut. In addition, IL-33 activates the expression of profibrotic genes, contributing to the development of intestinal fibrosis (upper panel). Different results are observed in chemically induced models of intestinal inflammation (middle and lower panels). During the onset of acute DSS-induced colitis, IL-33, likely released by necrotic/damaged epithelial cells, participates in the development of intestinal inflammation with a potent chemotactic effect on neutrophils, cells that play a pivotal role in this specific model of colitis (middle panel). Conversely, during the recovery phase of chronic DSS colitis, IL-33 appears to promote wound healing, inducing the restoration of epithelial barrier integrity. On the contrary, when IL-33 is administered to mice displaying a TNBS-induced colitis, which is mainly driven by a Th1 immune response, IL-33 shows an anti-inflammatory effect as a result of the skewing towards a Th2 immunophenotype and the potential induction of T regulatory cell activation (lower panel).

tissue/organ systems, such as the airways/lungs or the joints, where inflammation, in itself, is the major detrimental agent to cause pathology. In more complex systems, wherein mucosal immune responses interact with a large bacterial load and epithelial barrier integrity and function is essential to consider, such as that found in chronic intestinal inflammation, the final outcome of this intricate interplay is difficult to predict and may vary according to slightly modifications

of the initial conditions. In fact, IL-33 appears to enhance intestinal inflammation in disease models, which are driven by Th2 and innate immune responses, such that observed in SAMP mice and the acute phase of DSS colitis, and possibly in UC patients. Conversely, IL-33's effects during a Th1-driven model, such as in TNBS colitis, may result in decreased intestinal inflammation mediated by cytokine and cell-mediated modulation of immune responses. On the other hand, emerging evidence suggests that IL-33 may have positive effects on epithelial repair and barrier function. High levels of IL-33 during acute inflammation are likely to worsen tissue damage, whereas they may enhance tissue repair during recovery, promoting wound healing and the restoration of the epithelial barrier, as shown in the DSS model. Thus, the initial features of the specific immune response and the timing of IL-33 administration/blockade may dictate the overall outcome of disease pathogenesis.

The nuclear localization sequence in IL-33's primary structure suggested a possible role and function for this cytokine as an "alarmin," that is, a protein released from dying/suffering cells as an extracellular sign of danger [79]. Consistent with this hypothesis is the fact that IL-33 is released by cells undergoing mechanical stress [80] and is cleaved by pro-apoptotic caspases into less active forms [10–12]. Moreover, data obtained on mononuclear cells confirm that IL-33 is overexpressed after TLR-2 and -4 stimulation, but it is released only when necrosis of these cells is induced [81]. On the same line, it has been shown that other danger signals, such as extracellular ATP, different pathogen-associated molecular patterns (PAMPs), and inflammasome activation, lead to the increased production of IL-33 in different cell types (i.e., glial cells, airway epithelial cells) [82–84]. Indeed, the "alarmin" paradigm may have the potential to reconcile how IL-33 possesses such a wide spectrum of effects in the gastrointestinal tract. That is, upon harmful stimuli, IL-33 is released by suffering epithelial barrier cells in order to recruit and activate immune cells and clear potential pathogens. At the same time, the presence of a defective and damaged epithelial barrier must be quickly repaired, and the need for prompt epithelial restitution and wound healing is promoted.

Interestingly, IBD genetic studies have identified a few candidate susceptibility genes, encoding proteins that are pivotal for the maintenance of epithelial cell integrity, such as the endoplasmic reticulum stress protein, proteins related to the autophagy process, and structural proteins [1]. It is tempting to speculate that when epithelial function is severely impaired as a consequence of mutations of the aforementioned genes, suffering intestinal epithelial cells may release high levels of IL-33, activating a potentially detrimental immune response. At the same time, the loss/dysregulation of ST2L on intestinal epithelial cells in IBD may alter epithelial restoration. As such, further, more mechanistic investigation is warranted to dissect this complicated scenario, aiming to clarify the specific effects of the IL-33/ST2 axis, at different times as well as the contribution of different cellular sources, in order to elucidate the predominant role of this complex cytokine system in the pathogenesis of intestinal inflammation.

Acknowledgments

The authors would like to acknowledge the continued support for their work from the National Institutes of Health (DK056762, DK091222/PPG4 and AI102269 to Theresa T. Pizarro), the Italian Ministry of University and Research (PRIN 2009NE3B5Z_004 to Maurizio Vecchi), and the Italian Society of Gastroenterology (SIGE) (Research Grant 2010-2011 to Luca Pastorelli).

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 391473, 9 pages
http://dx.doi.org/10.1155/2013/391473

Review Article

Pre- and Posttherapy Assessment of Intestinal Soluble Mediators in IBD: Where We Stand and Future Perspectives

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Received 19 January 2013; Accepted 3 April 2013

Academic Editor: David Bernardo Ordiz

Inflammatory bowel disease (IBD) is a chronic inflammatory condition characterized by an abnormal immune response against food or bacterial antigens in genetically predisposed individuals. Several factors of innate and adaptive immune system take part in the inflammatory process, probably actively contributing in endoscopic and histological healing at molecular level. Although it is difficult to discriminate whether they are primary factors in determining these events or they are secondarily involved, it would be interesting to have a clear map of those factors in order to have a restricted number of potentially "good candidates" for mucosal healing. The present review will present a class of these factors and their modulation in course of therapy, starting from pathogenic studies involving several treatments associated with good clinical outcomes. This approach is meant to help in the difficult task of identifying "good candidates" for healing signatures, which could also be possible new therapeutic targets for clinical management of IBD patients.

1. Introduction

Inflammatory bowel diseases (IBDs) are chronic inflammatory conditions characterized by chronic intestinal mucosa damage caused by an abnormal immune response against food or bacterial antigens [1–3]. New therapies, including biologics, have been proved to induce mucosal healing in both Crohn's disease (CD) and ulcerative colitis (UC) [1, 2]. Mucosal healing has been associated with reduced hospitalization, maintenance of remission and better clinical outcomes [4, 5].

Mucosal healing in course of IBD is still unclear, starting from its definition. The most commonly known definition of mucosal healing is the "endoscopical" healing, whose quantification has been made possible through endoscopic scores, as Mayo score 7 [6], SES-CD [7], and Rutgeerts' score (especially used to assess the endoscopic recurrence of CD in patients undergone to surgical interventions) [8].

Endoscopic healing could be related to an immunological repair of lesions; however a clear dependence of endoscopical healing on "histological healing" has not been found, and furthermore it has not been fully explored the relationship between endoscopy, histology, and biological repair [9].

Starting from this hypothesis, many studies tried to find molecular signatures to predict the trend of the disease, searching within the key players of IBD pathogenesis, such as pathways related to mucosal permeability and response to environmental agents [10], genetic factors, as genes involved in intracellular pathogen recognitions (*NOD* [11]), autophagy not mitochondria or chaperone associated (*ATG16L1* [12], *IRGM* [13, 14], or *LRRK2* [15]), cytokines receptor (*IL-23* [11]), or ER stress unfolded protein response elements (*XBP1* [16–18], *AGR2* [19], and *ORMDL3* [20–22]), and genes related to adaptive and innate immune responses.

Products of genes related to adaptive and innate immunity have been extensively associated with injured tissue, where chronic inflammation is sustained by an activation

of mast cells/macrophages, neutrophils, and dendritic cells, followed by activation of T cells and other leukocytes.

Both animal studies and human studies have led to the identification of different subpopulations of T cells that are activated in an aberrant manner, respectively, in CD (Th1 and Th17) [23–25] and UC (Th2) [26].

While mucosal healing, and probably "histological healing," is becoming a reality close related to clinical practice, the "biological healing," associated with an immunological restoration in gut mucosa, is until applied only in research field, usually in studies assessing the response to a certain therapy in course of IBD.

Aim of this narrative review is to collect good examples of experimental evidences correlating endoscopic, histological, and biological healing in course of IBD, in response of well-defined therapeutic interventions (Table 1). Therapies will be divided into drugs acting systemically or locally with a broad spectrum of action and systemic drugs with a specific target.

2. Systemic or Topical Drugs with Broad Spectrum of Action

2.1. 5-Aminosalicylic Acid/Sulfasalazine. Mesazalazine (5-ASA) is one of sulfasalazine derivatives. It is administered starting 2 g/die until 4.8 g/die particularly in UC patients, but also in patients affected by CD [23]. It is produced in tablets, which deliver the active form in colonic mucosa in a pH-dependent or time-dependent way. New formulations of 5-ASA involve a different system able to increase colonic release [24, 25]. Mesalazine also acts locally, being active on rectum and left colon [27]. 5-ASA action depends on its ability to inhibit in vitro leukotriene (LT)B4 and prostaglandin (PG)E2 production. These effects were evaluated in biopsy specimens grown in culture for 24–48 h from healthy and/or UC or CD patients [28]. Other reports show that 5-ASA could decrease IL-1beta production during a 24-hour treatment of biopsy samples from patients with active IBD [29, 30] or inhibit the activation of NF-kB [31], so decreasing the expression of several cytokines (TNF, IL-1, IL-2, IL-6, and IL-8) or adhesion molecules (ICAM-1, VCAM-1, E-selectin, and MAdCAM-1) and enzymes involved in inflammation, like inducible nitric oxide synthase and cyclooxygenase-2 [32, 33]. In 2000, Bantel et al. showed that endoscopic healing in 20 UC patients treated with 5-ASA correlated with a reduced expression of NF-kB at immunohistochemical staining on tissue sections [31]. Elevated levels of LTB4 have been reported in colonic tissue from patients with UC: lipid extracted was analyzed by high-pressure liquid chromatography and biopsy specimens from patients affected by IBD and contained 254 ng of LTB4 per gram, in comparison with mucosa from normal subjects containing less than 5 ng of leukotriene B4 per gram of biopsy weight [34–36]. 5-ASA has shown ability to induce peroxisome proliferator-activated receptor γ (PPAR-γ) in vitro on HT29 colon epithelial cell line [37]. This result correlates with the finding that PPAR-γ, at mRNA and protein levels, is lower on specimens from patients affected by UC compared to CD or controls, and that use of and response to 5-ASA were associated with a reestablishment of its levels [38]. In these patients, the effect was observed only at intestinal mucosal levels and not within peripheral blood mononuclear cells, suggesting that these changes were depending on epigenetic alterations induced by the drug at mucosal level [38]. PPAR-γ is a nuclear receptor that activates kinases and other transcription factors implicated in inflammatory process such as nuclear factor kB (NFkB), c-Jun, c-Fos, and nuclear factor of activated T cell (NFAT) [39–41] and inhibits mucosal production of inflammatory cytokines (IL-1β and TNF-α) and chemokines [42], proliferation of inflammatory cells [43], and expression of some adhesion molecules [44].

2.2. Corticosteroids. Steroids are among the most potent anti-inflammatory drugs known in human pharmacology and the most widely bioavailable: their lipophilic characteristics allow corticosteroids to passively diffuse across cellular phospholipid layer and to bind their cytoplasmic receptors expressed in every tissue [45]. The corticosteroid receptor is a member of the nuclear receptor (NR) superfamily, which includes receptors of other hydrophobic molecules like biliary acids, A and D vitamins' and thyroid hormones. NR superfamily shares in common the same structure with three functional domains: at N-terminal part they present a transactivation domain; at C-terminal part there is a ligand-specific binding protein (LBD); between two terminus there is a central zinc finger DNA-binding domain (DBD) which binds specific DNA sequences, termed glucocorticoid-responsive elements (GRE) [46]. This interaction allows the increase of lipocortin 1 syntesis, a phospholipasis A2 inhibitor, inhibition of arachidonic acid release [47], and the increase of IkBa expression which binds NFkB and maintains it inactive [44–46]. Moreover, the heterocomplex corticosteroid-receptor inhibits some interactions among transcriptional factors and their specific genes, like the inhibition of link between NFkB and cytokines sequences [44–46]. Different kinds of steroids are known, and their anti-inflammatory power is usually related to cortisone derivatives [48]. Steroids can act on all cells of our body, particularly immune cells, and that is, probably, the main reason of their efficacy on IBD [49]. Recently, poorly absorbable steroids, active only at mucosal levels, have been shown efficacy in treatment of IBD [50–53]. Because of their structures they are believed to act through the same pathways as systemic steroids, although directly on intestinal mucosa [54]. Active treatment with corticosteroids has reduced activation of NFkB in colonic biopsy of 13 IBD patients as detected by electrophoretic mobility shift assays, following 3 weeks of treatment with 0.75 mg/kg per day prednisolone. [51]. Moreover, some studies also showed a dose-dependent inhibition of intestinal epithelial cell migration and proliferation in bowel, especially prednisolone, budesonide, and dexamethasone at lower concentrations [52, 53, 55]. In vitro studies on intestinal mucosa from IBD patients showed that treatment with dexamethasone lowered levels of IL-1beta and leukotriene B4 [56]. In a recent paper [57] it was shown that in CD patients use of steroids, as well as immune-suppressant and anti-TNF-α drugs, was associated with downregulation of MMP-9 and MMP-26 positive neutrophils and stromal TIMP-1 and TIMP-3 and

Table 1: Effects of therapeutic strategies in IBD patients.

(a) Crohn disease

Treatment	5-ASA	Corticosteroids	AZA; CsA	IFX	ADA
Method	HPLC; ELISA; RT-PCR	ELISA; RT-PCR	ELISA; RT-PCR	ELISA; RT-PCR	ELISA; RT-PCR
Target	Protein; nucleic acid	Protein; nucleic acid	Protein; nucleic acid	Protein; nucleic acid	Protein; nucleic acid
Major findings	↓IL-1beta	↑lipocortin 1	↑T-cells apoptosis	CD40,CD40L, and VCAM-1	=TNF-α and IL-17A
	↓TNF-α, IL-1, IL-2, IL-6, and IL-8	↑IkBa	↓Rac1	↑T-cells apoptosis	↓IL23, IFN-γ, and IL-10
	↓ICAM-1, VCAM-1, E-selectin, and MAdCAM-1	↓NF-κB	↓(MEK), bcl-x(L)	↑CD4 + CD25highFoxp3+ T cells	
	↓NF-κB	↓MMP-9, MMP-26, TIMP-1 and TIMP-3	↓NF-κB	↑Plasma TNF-α	
	↑PPAR-g	↓ IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, TNF-α	↑G2 cell cycle arrest	↓SerumIL-6	
	↓LTB4	↓Intestinal epithelial cell migration and proliferation	↓Calcineurin activation	= IL-23, IL-17A, and INF-γ	
	↓PGE2	↑Neutrophil survival	↓NFAT		
		↓hGC	↓IL-2		
References	[32, 34–50]	[54–68]	[69–72]	[73–78]	[79]

(b) Ulcerative colitis

Treatment	5-ASA	Corticosteroids	AZA; CsA	IFX	ADA
Method	HPLC; ELISA; RT-PCR	ELISA; RT-PCR	ELISA; RT-PCR	ELISA; RT-PCR	IHC;ELISA; RT-PCR
Target	Protein; nucleic acid	Protein; nucleic acid	Protein; nucleic acid	Protein; nucleic acid	Protein; nucleic acid
Major findings	↓IL-1beta	↑lipocortin 1	↑T-cells apoptosis	CD40,CD40L, and VCAM-1	=TNF-α and IL-17A
	↓TNF-α, IL-1, IL-2, IL-6, and IL-8	↑IκBa	↑G2 cell cycle arrest	↑T-cells apoptosis	↓IL23, IFN-γ, and IL-10
	↓ICAM-1, VCAM-1, E-selectin, and MAdCAM-1	↓NF-kB	↓Calcineurin activation	↑CD4 + CD25highFoxp3 + T cells	↑Notch-1
	↓NF-κB	↓ IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α	↓NFAT	↓TNF-α and IFN-g m-RNA	
	↑PPAR-g	↓Intestinal epithelial cell migration and proliferation	↓IL-2	= IL-10 and IL-4 mRNA	
	↓LTB4	↑Neutrophil survival			
	↓PGE2	↓hGC			
References	[32, 34–50]	[54–68]	[69–72]	[80–82]	[79, 83]

this paralleled histology score and calprotectine. Furthemore, Raddatz et al. analyzed systematically several cytokine mRNA expressions in intestinal mucosa from IBD patients in following oral steroid therapy [58]. IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α were evaluated by quantitative reverse transcriptase-polymerase chain reaction in biopsies and PBMNC, and their changes correlated with endoscopic findings, clinical activity, and outcome after 6 months from therapy. Among all cytokines, IL-1β, IL-6, and TNF-α were the most represented, but, in contrast to IL-1β and TNF-α, IL-6 expression was restricted only to inflamed mucosa and correlated better with clinical activity and C-reactive protein levels. Corticosteroids are reported to suppress the levels of cytokine mRNA [59] although they have a paradoxical action of promoting neutrophil survival [60, 61]: therapy with dexamethasone induces eosinophil apoptosis, but it is a great inhibitor of neutrophil apoptosis. Another interesting paper suggested that side effects associated with steroid treatment

were associated with an heavy depression of NF-kB activity, which in normal conditions regulates human glucocorticoid (hGC) receptor-l levels in an autoregulative molecular loop [62].

2.2.1. Azathioprine and Cyclosporine. These are among the most used immunosuppressants in IBD. Azathioprin (AZA) therapy was one of the first drugs described to lead to mucosal healing in CD patients [63]. AZA/6-mercaptopurine is an inosin analogue that decreases acid nucleic synthesis especially in lymphocytes, with a decrease of immune responder cells [54]. It was reported that AZA therapy in absence of corticosteroids led to endoscopic mucosal healing in 73% of 19 patients after 6 months [64], while inducing T-cells apoptosis [65]. Moreover, azathioprine is able to maintain mucosal healing in contrast with corticosteroid therapy [63]. Data about treatment of UC with AZA therapy is controversial, especially about the maintenance of remission [66]. In vitro studies analyzing the effects of the drug on T cells from lamina propria of colonic specimens from CD patients, showed that it was able to suppress Rac1 activity genes, like mitogen-activated protein kinase kinase (MEK), NF-kB, and bcl-x(L), leading to a mitochondrial pathway of apoptosis. AZA treatment is associated with disappearance of the inflammatory infiltrate [8] and G2 cell cycle arrest [67].

Cyclosporin (CsA) blocks the signaling transduction as it does not allow the calcineurin activation. This is associated with the lack of activation of the transcription factor NFAT and consequently the transcription on IL-2. The CsA pathway is very important in T cells because of their dependency of IL-2 to organize their immune responses [54]. Although there is not a specific effect of CsA on mucosal healing [68], in acute severe UC cyclosporine could be a powerful rescue therapy for patients not responding to steroid treatment [84]. A recent study [85] showed that use of cyclosporine does not avoid colectomy in 50% of subject, as it was not associated with induction of mucosal healing in these patients.

2.3. Systemic Drugs with Specific Targets: Anti-TNF. Recombinant techniques and improvements in molecular biology field allowed to create totally human or humanized monoclonal antibodies, in vitro anti-human cytokines, such as TNF-α, and antimembranous proteins like integrins, or phenotype proteins, like anti-$\alpha 4\beta 7$ antibodies, called "biologic agents." The production of these molecules allowed to hit specific molecular targets, in order to escape side effects of more wide-spectrum drugs and resulted in a better understanding of the pathophysiology of diseases, and it has been made possible through *phage-display* methods [69].

Drugs actually used in IBD include infliximab, adalimumab, and other molecules still under development or in clinical trials.

2.3.1. Infliximab (IFX). It is a chimeric monoclonal antiboby, not fully human, with variable regions of Fab with murine origin, directed to human TNF-α. It was first released for CD and then for UC therapy. Together with clinical improvement, it has been described that IBD patients receiving IFX showed a decrease of intestinal permeability.

Use of IFX is associated with lowering of microvascular CD40 and VCAM-1 expression in mucosal biopsies evaluated by immunohistochemistry [50]. In the same study the same changes have been showed in serum levels of plasmatic sCD40L and platelet/peripheral blood T-cell (PBT) CD40L expression.

One of the most important mechanisms of action of IFX is the induction of T-cells apoptosis. It has been shown, in fact, that T cells, isolated by CD3 selection from IBD patients after treatment in vitro with IFX, go to apoptosis [70]. Treatment with IFX was associated with a reestablishment of regulatory T cells ($CD4^+CD25$ + $Foxp3^+$ T cells or T regs) in intestinal mucosa as well as in peripheral blood, particularly, in responder patients, together with a decrease of their apoptosis [71]. A study (ACCENT I) has demonstrated that mucosal healing occurred in 9 of 10 patients with CD after four weeks of a single infusion of IFX [72]. In this kind of patients an endoscopic substudy on ACCENT I showed mucosal healing in 99 patients: the patients that received 3 infusions of infliximab (0-2-6 weeks) demonstrated mucosal healing compared to the patients that received only 1 infusion of infliximab [86]. A recent study showed the changes of circulating cytokines in CD responder and not-responder patients in IFX treatment during endoscopic evaluation. A significant increase in the plasma TNF-α level was found at week 6 in both groups and in contrast, at both week 2 and week 6 the serum IL-6 levels tended to be lower than at baseline [87]. The serum levels of other cytokines (IL-23, IL-17A, and INF-γ) did not show significant changes. The authors of the same study hypothesized that the same TNF-α stimulated the cytokine production (such as IL-23, IL-12p70, IL-17, and IL-6 syntheses by LPMCs in CD patients [88]) and IFX, blocking TNF-α, inhibited their production.

The trials ACT1/2 showed that the use of IFX in patients with UC, already at week 8, was associated with mucosal healing, as indicated by an endoscopic subscore 0-1 compared to baseline at week 0, and that was paralleled to a lower risk of colectomy over the next year [73]. In 2009, an interesting study showed that in patients affected by UC following the induction phase with IFX (5 mg/Kg), TNF-α and INF-γ mRNA levels in colonic biopsies lowered, but not those of IL-10 and IL-4. Furthermore, decrease in TNF-α mRNA was correlated with clinical and endoscopic improvements [74].

In another paper assessing 35 CD patients before and after 2 or 6 weeks from starting IFX therapy [87], it was shown that higher levels of IL-17A, IL-23, and IL-12 at baseline were predictive for lower therapeutic response to IFX therapy, as their levels remained high also after therapy.

Biopsies from UC patients treated in vitro with IFX showed that IFX induced a reduction neither in TNF-α-mRNA nor of IL-1β-mRNA, but of IFN-γ-mRNA and, in a lower extent, of IL-6-mRNA [75].

2.3.2. Adalimumab (ADA). It is a full human antibody against TNF-α, licensed for both UC and CD. A prospective study showed how ADA was able to induce endoscopic healing and normalization of mucosal cytokine investigated

by mRNA expression in patients with active CD [76]. This study included 77 patients and they were examined by endoscopy before and after therapy (with a minimum of six ADA injections). Biopsies were collected for measurements of mRNA expression levels of IL-17A, IL23, IFN-γ, TNF-α, IL10, and Foxp3, as well as for immunohistochemistry. Complete endoscopic healing was achieved in 27.3% of patients after 10 weeks of treatment and it was associated with a significant reduction in mRNA expression levels for all cytokines except IL10. Elevated expression of TNF-α and IL-17A persisted in 52% and 76%, respectively, of patients with complete endoscopic remission. Pretreatment cytokine gene expression levels did not predict response to ADA therapy. A study about T-cells apoptosis showed ADA was able to induce it increasing the Notch-1 pathway [77]: by immunohistochemical staining, lower levels of Notch-1 were detected in UC inflamed mucosa and it increased in response to anti-TNFα treatment. This observation has an important immunological significance as Notch-1 inhibition prevents T-cell cycle arrest (induced by anti-TNF-α) but not apoptosis.

3. Methodology Used to Assess Immunological Signatures in IBD

As suggested from the above-reported studies, various techniques have been used for different experimental approaches. They can be overall divided into ex vivo studies and in vitro studies and for protein and acid nucleic analyses. The first group, easier to perform, comprehends direct techniques able to characterize immunological signatures on biologic samples fixed in formalin or frozen.For analysis of proteins of outer membrane or cytoplasmic, the most diffused techniques include immunohistochemistry and western blotting, while for nucleic acid analysis, real-time PCR [78], mRNA microarray, and tissue microarray [80]. The greater advantage of microarray is the possibility to screen in the same time several mRNA or protein: cDNA or oligonucleotides are spotted on the slide surface [81, 82]. Tissue microarrays (also TMAs) are paraffin blocks with until 1000 different separate tissue cores: they are assembled in arrays to allow multiplex histological analysis [79, 83]. The great limitation of this tool is that it does not show which part of the tissue is expressing that particular protein. Immunohistochemistry and fluorescent in situ hybridization (FISH), on the contrary, could be complementary to them. Ex vivo study is usually a "static study," as the above methods report a "picture" of the proteic state of a patient gut mucosa, in a precise moment. Western blot has a good sensitivity, able to discriminate easily a negative compared to a positive result, but it does not allow to localize a specific protein in a tissue, as it starts from protein lysate of the biological specimens like colonic biopsies.

mRNA analysis by RT-PCR is another very valid method to indirectly evaluate protein levels. It could be used to evaluate a response state of a tissue at different time points.A second group of techniques are dealing with in vitro studies. Many experiments described in this review have been performed by in vitro studies consisting in culturing colonic biopsies or colonic cell lines [29, 89]. The major advantage of these studies is the possibility to work dynamically. Besides methods already described above, this approach allows measurement of released cytokine by ELISA assay, Multiplex assay, and flow cytometry, if they start as membrane proteins [90]. With the last method it is possible to evaluate cellular apoptosis or the nuclear expression of $CD4^+CD25^+Foxp3^+$ T regulatory cells [91]. Multiprotein ELISA assay is a test that allows to measure up to tens of cytokines at the same time from the same serum or tissue culture supernatants or other biological fluids: it is characterized by a particular technology called Xmap technology [92]. Recently this assay was used to evaluate the serum cytokines profile in UC patients [93]. New approaches, already well utilized in oncobiology research, are the micro-RNAs or miRNAs [94, 95] detection by real time PCR [96, 97]. miRNAs are single-strand oligonucleotides that bind mRNA and disrupt it in cytoplasmatic bodies called P-bodies. This effect inhibits cellular ribosome-associated transduction and protein synthesis. To date, however, specific miRNA controlling production of cytokines is still not completely clarified.

4. Conclusions

Endoscopic procedures remain the first line to evaluate response to therapy as well as to assess endoscopic state of the diseases. Mucosal healing has been associated, particularly for studies assessing efficacy of biologic therapy, with amelioration in clinical outcomes like hospitalization or surgery, for both UC and CD. Several studies suggest that mucosal healing or amelioration of mucosal inflammation and clinical outcomes correlate with several changes in mucosal immunity. The majorities of changes registered are related to a reduction in proinflammatory molecules levels or to the reduction in activation of transcription factors such as Nf-KB. A broader effect on mucosal immunity seems to be related to use of steroids, by reduction of several cytokines such as TNF-α, IL-1, IL-2, IL-6, and IL-8, for both UC and CD. Use of biologics, particularly IFX, is associated with a modulation of both cytokines expression as well as other important immune components of gut mucosa, such as regulatory T cells. The effect of immunosuppressants, particularly AZA and Csa, is related to their primary effect on immune cells. 5-ASA induces a reduction in integrin expression on mucosal endothelial cells, similarly to what is observed for biologics, particularly IFX. Despite the heterogeneity of different studies reported, different drugs could produce similar results in terms of modulation of selected cytokines or inflammatory pathway. That could probably reflect a common pathway of action of different drugs, or, more likely, the same positive response for a patient, for a therapeutic intervention. These observations open new important consideration on mechanisms of action of different drugs, very often not well known, and secondarily, to the mechanism of healing processes which could share similar pathway between different treatments. When "normal" healing responses are not generated, disequilibrium of cytokine content is observed among patients, that could relate to different and not exhaustive responses to certain drug. These studies open new perspective on discovery of biological and tissue-specific prognostic factors in IBD therapy. As an

example, Matsuda et al. showed that patients not responding to therapy were displaying higher levels of TNF-α and IL10 in course of UC [98], while higher levels of mucosal TNF-α and IL-6 were observed in nonresponding CD [88]. Despite interesting findings, major limits reduced the applicability of these examinations. One of the first reasons is that the majority of studies available are only observational studies. To the best of our knowledge, there are no trials assessing as an endpoint the "biological response," instead of clinical or endoscopic response or the prospective validity of the mucosal biomarker for both CD and UC. Furthermore, a common, standardized method of analysis used to establish a potential cut-off of reported values is also not available. Finally, whether "old" or classic techniques available are more reliable, widely diffused, and able to assess few targets at the time, new emerging techniques, like microarray analysis or miRNA analysis, display a broader potential to picture the immune and metabolic status of gut mucosa. A broader analysis, however, is more difficult to interpret and mathematic clustering of data still does not correspond to validated or standardized immune-metabolic phenotype, useful for daytime practice. Further studies assessing immune signatures in response to therapy are warmly welcome, particularly those assessing new mechanisms of action of clinical efficacious drugs. This approach could identify good candidates for mucosal prognostic biomarkers, together with new therapeutic targets for future researches.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 515048, 7 pages
http://dx.doi.org/10.1155/2013/515048

Research Article

Increased Expression of VEGF and CD31 in Postradiation Rectal Tissue: Implications for Radiation Proctitis

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Received 15 January 2013; Revised 14 March 2013; Accepted 15 March 2013

Academic Editor: David Bernardo Ordiz

Background. Inflammation mediators related to radiation proctitis are partially elucidated, and neovascularization is thought to play a key role. *Objectives*. To investigate the expression of vascular endothelial growth factor (VEGF) and CD31 as angiogenetic markers in postradiation rectal tissue. *Methods*. Rectal mucosa biopsies from 11 patients who underwent irradiation for prostate cancer were examined immunohistochemically for the expression of VEGF and CD31 at three time settings—before, at the completion of, and 6 months after radiotherapy. VEGF expressing vascular endothelial cells and CD31 expressing microvessels were counted separately in 10 high-power fields (HPFs). VEGF vascular index (VEGF-VI) and microvascular density (MVD) were calculated as the mean number of VEGF positive cells per vessel or the mean number of vessels per HPF, respectively. Histological features were also evaluated. *Results*. VEGF-VI was significantly higher at the completion of radiotherapy (0.17 ± 0.15 versus 0.41 ± 0.24, $P = 0.001$) declining 6 months after. MVD increased significantly only 6 months after radiotherapy (7.3 ± 3.2 versus 10.5 ± 3.1, $P < 0.005$). The histopathological examination revealed inflammatory changes at the completion of radiotherapy regressing in the majority of cases 6 months after. *Conclusions*. Our results showed that in postradiation rectal biopsy specimens neoangiogenesis seems to be inflammation-related and constitutes a significant postradiation component of the tissue injury.

1. Introduction

Irradiation is an important adjuvant therapy in the treatment of pelvic malignancies. However, it often results in collateral damage to the surrounding of the primary tumor site. The most frequent complication after radiotherapy treatment for prostate cancer is radiation proctitis with incidence rates ranging from 2% to 39% [1]. Radiation damage may occur in acute or chronic form. Acute radiation proctitis occurs immediately or up to 3 months after the initiation of radiotherapy. The presenting symptoms are diarrhea, tenesmus, urgency, mucus discharge, and bloody stools. In contrast, chronic proctitis may appear years after the completion of therapy usually manifesting by gross hemorrhage. The newer irradiation modalities—3D conformal radiotherapy and intensity-modulated radiotherapy—are usually not associated with severe side effects such as ulceration, fistulation, necrosis, and stricture, but bleeding due to radiation proctitis still occurs even at a lower rate. Radiation proctitis is diagnosed by endoscopy where edematous, friable, and with abnormal telangiectatic vessels mucosa is usually demonstrated [1, 2].

The pathophysiology of radiation proctitis is only partially elucidated, and several mechanisms have been put forward. The earliest studies suggested that blood vessels are the main site of injury and that microvascular compromise is an important factor in the natural history of radiation proctitis.

The pathogenetic process triggered by radiation seems to be multifactorial including several molecular events leading to inflammation, hypoxia, neovascularization, and fibrosis [3–5]. Various cytokines and growth factors have been implicated in the pathogenesis including hypoxia-inducible factor 1 (HIF-1), transforming growth factor β1 (TGF-β1), fibroblast growth factor 1 (FGF-1), and vascular endothelial growth factor (VEGF) [6–8]. Radiation-induced inflammatory response is closely related to oxidant stress, while an increase in free oxygen radical production has been documented as a result of infiltrating inflammatory cells and of radiation-induced ischemia [9, 10].

Angiogenesis plays an important role in many chronic inflammatory diseases, while VEGF is the main stimulatory factor [11]. It is secreted by macrophages, endothelial cells, fibroblasts, smooth muscle cells, and activated platelets. VGEF induces proliferation, inhibits apoptosis of endothelial cells, increases vascular permeability, and has a chemotactic effect on macrophages. VEGF gene expression is regulated by various mechanisms the most important being hypoxia, especially through upregulation of HIF [12, 13].

There is very limited and conflicting experimental data regarding the role of angiogenesis in the context of postradiation proctitis, especially in the chronic form of the disease. The information derives mainly from mice studies where a gradual increase of VEGF after an early peak of HIF was observed in rectal tissues during the first 3 months after their irradiation [6]. As for humans, an increased angiogenesis not corresponding to a VEGF overexpression has been demonstrated in cases of radiation proctitis at 7–38 months after irradiation [7].

The aim of our study was the immunohistochemical investigation of angiogenesis in postradiation rectal mucosa in association with VEGF expression and in relation to histological findings in the early and late postradiation period in order to provide some evidence regarding the involvement of neoangiogenesis in mucosal injury.

2. Materials and Methods

2.1. Sample Selection. We prospectively enrolled consecutive patients with prostate cancer who were newly referred and treated with 3D conformal radiotherapy schedule of 72–74 Gy of total dose. In all patients, the total dose did not exceed 70 Gy for more than 25% of rectal volume. Four rectal biopsies were obtained randomly at sigmoidoscopy from normal-appearing mucosa at least 1 cm away from any macroscopically-visible lesion using a 6 mm forceps. The endoscopy was performed before, at the completion of (within an interval of 1–3 days), and 6 months after radiotherapy. None of the individuals had a personal history of colorectal cancer, and all had the same bowel preparation. The study was approved by the hospital's ethics committee, and an informed consent was signed by every individual before the procedures.

2.2. Histology and Immunohistochemistry. All biopsy specimens were fixed in formalin solution and processed according to routine protocol. Four μm thick paraffin sections were stained with hematoxylin and eosin for histological assessment and for immunohistochemical analysis. The following primary antibodies were applied: (i) monoclonal mouse anti-human CD31 antibody (MO823; Dako, Glostrup, Denmark) at a dilution 1 : 50 and (ii) monoclonal mouse anti-human VEGF antibody (M7273; Dako, Glostrup, Denmark) at a dilution 1 : 50. Detection was carried out using the DakoEnvision Detection System Peroxidase/DAB+ (K4065; Dako, Glostrup, Denmark).

2.3. Histological Examination and Assessment of Immunostaining. Histological evaluation included inflammatory infiltrates, presence or absence of cryptitis and of crypt abscesses, erosions or ulceration, and thickening of the subepithelial collagen plate.

For the evaluation of immunostaining, 10 high-power fields were examined arbitrarily under ×400 magnification for each case. VEGF positive vascular endothelial cells and CD31 expressing microvessels were counted. VEGF "vascular index" (VEGF-VI) and "microvascular density" (MVD), that is, the mean number of VEGF positive cells per vessel or the mean number of vessels per field, were calculated, respectively. The vessels with staining of CD31 were counted to examine MVD. Average of all fields was used for the analysis of VEGF and MVD. Immunohistochemical assessment was performed by two examiners, blinded to clinical information. For each case, agreement was reached by simultaneous evaluation of the specimens using a two-headed microscope.

2.4. Statistical Analysis. Statistical analysis was performed using the statistical package Statgraphics Centurion XV (StatPoint Technologies, Inc. Corp. Warrenton, VA, USA). Results in the text are presented as mean with standard deviation. The paired t-test was used to compare differences between groups. A P value <0.05 was considered significant. Data in figures are presented as box-and-whisker plots. The box includes 50% of the results falling between the 25th and 75th percentile (interquartile distance). The median value is represented as a horizontal line inside the box. Outliers, that is, points more than 1.5 times the interquartile range from the end of the box, are shown as open squares.

3. Results

Eleven patients with a mean age of 72.4 ± 10.5 years were studied. Three additional patients missed to followup and were excluded from the analysis. During the followup period, 2 patients reported rectal bleeding that settled without specific intervention, while in 4 patients endoscopy revealed the presence of mild to moderate radiation proctitis not needing a therapeutic intervention according to endoscopic classification by Zinicola et al. [14].

3.1. Histology. Preirradiation biopsies showed normal rectal mucosa in all 11 patients. Histological findings in the early postirradiation period were predominantly characterized by inflammatory changes. In 6 cases, inflammatory changes were consisted with mild active colitis characterized by infiltration of the lamina propria mainly by neutrophils, mild cryptitis, and few crypt abscesses. Small telangiectasias were found only in one case, while fibrin microthrombi were not

Table 1: Histological findings in an individual patient at three time settings—before, at the completion of, and 6 months after radiotherapy.

	Before radiotherapy	At the completion of radiotherapy	6 months after radiotherapy
Patient 1	Normal	Mild active colitis-mild focal crypt atrophy-focal fibrosis	Mild nonspecific chronic inflammation
Patient 2	Normal	Focal fibrosis-mild crypt distortion	Mild nonspecific chronic inflammation-mucinophages
Patient 3	Normal	Focal fibrosis-mild focal crypt atrophy-thickening of the subepithelial collagen band	Mild nonspecific chronic inflammation-mild focal fibrosis-mucinophages
Patient 4	Normal	Focal fibrosis-mild focal crypt atrophy-thickening of the subepithelial collagen band	Mild nonspecific chronic inflammation
Patient 5	Normal	Mild active colitis	Mild nonspecific chronic inflammation-few mucinophages-focal fibrosis
Patient 6	Normal	Mild active colitis	Mild nonspecific chronic inflammation
Patient 7	Normal	Mild active colitis-few angiectasis	Mild nonspecific chronic inflammation
Patient 8	Normal	Mild active colitis-focal fibrosis	Thickening of the subepithelial collagen band-few mucinophages
Patient 9	Normal	Mild active colitis-thickening of the subepithelial collagen band	Focal crypt atrophy
Patient 10	Normal	Focal fibrosis-mild focal crypt atrophy-mild crypt distortion	Mild nonspecific chronic inflammation-few mucinophages
Patient 11	Normal	Mild crypt distortion	Focal fibrosis-thickening of the subepithelial collagen band

Table 2: Sequential change of VEGF and CD31 in an individual patient at three time settings—before, at the completion of, and 6 months after radiotherapy.

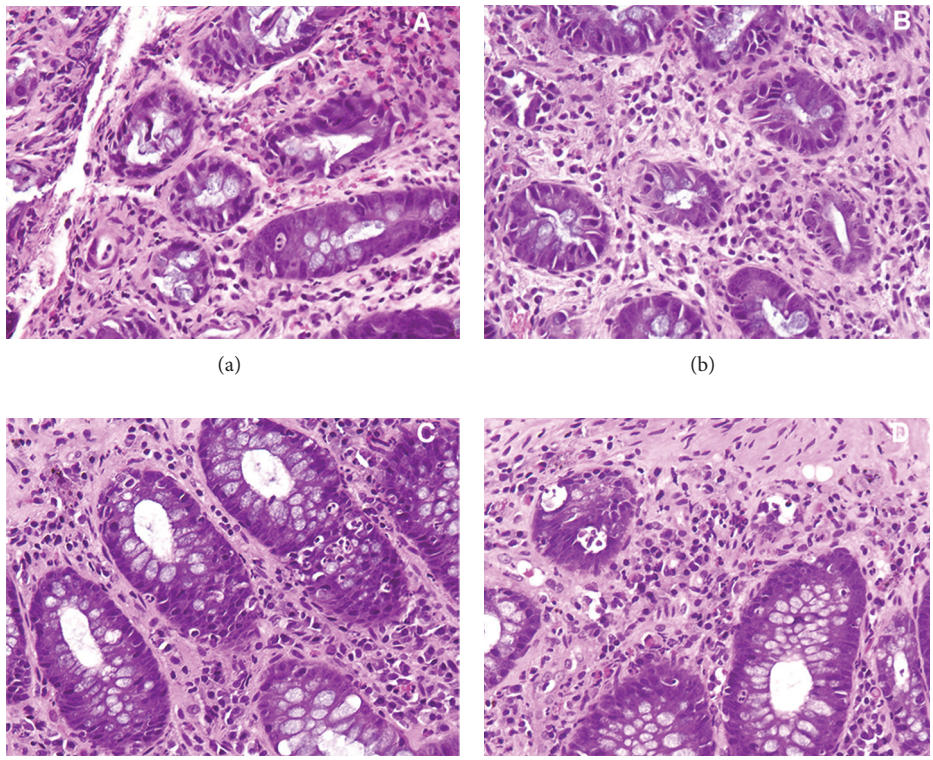
Patient	CD31_pre_radio	CD31_at completion	CD31_6 months after	VEGF_pre_radio	VEGF_at completion	VEGF_6 month after
1	7.7	7.2	4.5	0.37	0.59	0.38
2	6.5	4.3	9.1	0.13	0.63	0.52
3	12.8	5.2	5.6	0.42	0.05	0.10
4	7.3	7.8	9.3	0.41	0.26	0.46
5	2.4	7.5	11.3	0.10	0.64	0.58
6	3.6	9.2	12.7	0.10	0.48	0.44
7	7.5	10.3	11.7	0.10	0.65	0.18
8	5.3	11.9	13.2	0.08	0.62	0.16
9	7.9	7.6	11.7	0.08	0.34	0.12
10	11.7	7.2	12.3	0.06	0.04	0.10
11	10.6	5.9	14.3	0.05	0.23	0.10

observed. Table 1 summarized the histological findings in an individual patient at the completion of radiotherapy.

In the late postirradiation period, the predominant diagnosis was mild nonspecific chronic colitis which was ascribed to 8 cases. Table 1 summarized the histological findings in an individual patient 6 months after completion of radiotherapy. The typical histological changes are presented in Figure 1. Histological findings such as crypt distortion, fibrosis, and vascular telangiectasia were limited probably due to the fact that our study was restricted to the "early" postirradiation period (first 6 months). Moreover, radiation tissue damage is expected to be less severe in relation to the contemporary radiation modalities.

3.2. Immunohistochemistry. Microvasculature was demonstrated by CD31 immunostaining, while VEGF was detected in endothelial cells and in few stroma cells showing cytoplasmic staining (Figure 2). Both VEGF-VI and MVD were increased at the completion of radiotherapy, the difference being significant only for VEGF-VI; 0.41 ± 0.24 versus 0.17 ± 0.15, $P = 0.001$ and 7.6 ± 2.2 versus 7.3 ± 3.2, $P = 0.61$, respectively. At the time of completion of radiotherapy, the mean values of VEGF and CD31 were significantly higher in cases with active colitis in histology examination compared to those showing no activity (0.55 ± 0.12 versus 0.24 ± 0.23, $P = 0.012$ and 8.95 ± 1.9 versus 6.08 ± 1.43, $P = 0.02$, resp.).

The increases of both indexes were also observed six months after the end of radiotherapy; the difference was significant for VEGF-VI and MVD compared to those before irradiation (0.29 ± 0.19 versus 0.17 ± 0.15, $P = 0.02$ and 10.5 ± 3.1 versus 7.3 ± 3.2, $P < 0.005$, resp.). VEGF-VI six months after the end of radiotherapy was significantly lower



(a) (b)

(c) (d)

Figure 1: Representative inflammatory epithelial changes in rectal tissue in the early postirradiation period consisting of infiltration of the lamina propria by neutrophils, cryptitis, and few crypt abscesses (c, d). Additional findings were mild focal fibrosis (a–d), mild crypt distortion, and atrophy (a, b).

compared to index at the time of radiotherapy completion (0.29 ± 0.19 versus 0.41 ± 0.24, $P < 0.05$). The results are summarized in Figure 3, while Table 2 presented the sequential change of VEGF and CD31 in an individual patient.

At the completion of the radiotherapy, there was a trend, which did not reach significance, for correlation between VEGF and CD31 ($P = 0.087$). The same, without statistical significance, trend was also observed after 6 months of radiotherapy completion ($P = 0.099$).

4. Discussion

Radiotherapy has been established as the treatment of choice for patients with prostate cancer. The use of conformal radiotherapy succeeded in reducing irradiation to organs at risk such as rectum, enabling a higher dose to the target volume [15, 16]. Even with this advanced technique, symptoms suggestive of postradiation rectum damage occur in up to 20% of patients depending on the dose and treatment method [17–19]. Acute symptoms observed early after external beam therapy of prostate cancer are thought to be mainly inflammatory in nature and settled spontaneously over few months after exposure in the majority of patients. However, in selected individuals postradiation reactions could be sustained for unclear reasons for months and even years after radiotherapy. This chronic form of radiation proctitis seems to be the result of submucosal inflammation, fibrosis, and angiogenesis [20].

Angiogenesis is a complex process mediated by multiple cells types and mediators, and besides its well-known role in cancer, it plays a critical role in hypoxic conditions and in several chronic inflammatory diseases [21]. Moreover, it potentiates the inflammatory response by increasing the influx of inflammatory cells as well as a chemotactic mediator [22, 23]. In our study, histological examination revealed an increased vascularity in rectal mucosa six months after radiation exposure. It was preceded by mucosal inflammation and concomitant VEGF expression appearing as early changes shortly after irradiation, while neither acute nor chronic ischemic lesions were found. These consecutive findings provide indications of a pathogenetic link between inflammation and vascularization, taking into consideration the higher values of VEGF and CD31 expression in patients with active colitis at the end of radiotherapy. The occurrence and/or persistence of newly formed microvessels after remission both of the inflammatory process and of the decrease of VEGF expression suggest a later postradiation and postinflammatory manifestation. Since now there are only few treatment options for patients with symptoms, such as bleeding due to radiation proctitis. Endoscopic treatment with argon plasma coagulation (APC) is considered the preferred treatment modality for radiation proctitis. Although APC successfully ameliorates symptoms

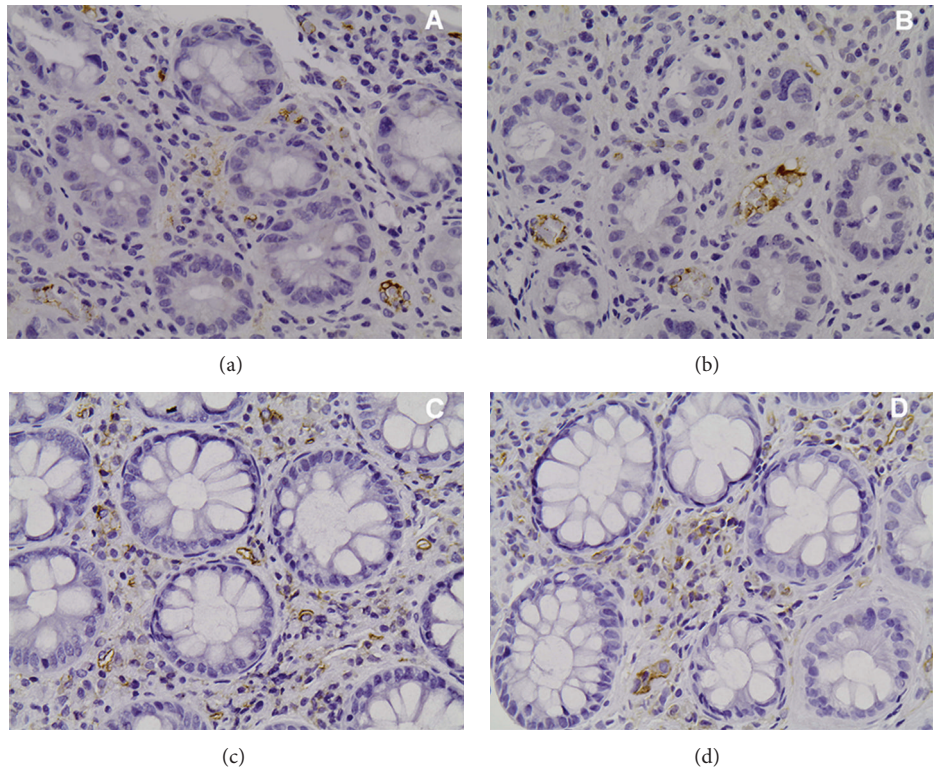


Figure 2: Rectal mucosa immunostained (a, b) with anti-VEGF antibody (M7273; Dako, Glostrup, Denmark) and (c, d) with anti-CD31 antibody (MO823; Dako, Glostrup, Denmark). Microvasculature was demonstrated by CD31 immunostaining of the vascular endothelial cells while VEGF was detected in endothelial cells and in few stroma cells showing cytoplasmic staining.

associated with mild endoscopic radiation proctitis, it is less effective in severe cases of the disorder. In these cases, intrarectal formalin—nevertheless an absolute toxic agent—is a useful therapeutic strategy [14, 24, 25]. According to our results, angiogenesis constitutes a component of mucosal injury in radiation proctitis; the clinical significance of new vessels formation following VGEF expression relies on the putative higher risk of bleeding complications. Thus, we could speculate on a possible effectiveness of antiangiogenetic drugs regarding the inhibition of excessive vascularization and the reduction of bleeding complications. However, the use of these compounds is restricted to cancer treatment and has been only experimentally investigated in colitis models and in IBD patients [26].

Our findings in the early postradiation period have demonstrated an association of increased VEGF expression with radiation-induced inflammation probably related to oxidative stress. This observation favors a beneficial impact of anti-inflammatory and antioxidant medication early in the course of postirradiation proctitis by preventing the initiation of inflammatory process directly after irradiation. Although someone could argue that there is no need to treat asymptomatic proctitis, it is well known to clinicians that asymptomatic radiation-induced proctitis is potentially a symptomatic one with or without rectal bleeding with an increasing time-related possibility. Thus, radiation-proctitis should be treated in a prevention manner, and our results showing early involvement of VGEF in the pathogenesis of this disease imply that the blockage of this factor could be a promising therapeutic option. Under this view, a combination of vitamin E (400 IU *tid*) and vitamin C (500 mg *tid*) has been proved a successful and sustained treatment of chronic radiation proctitis [27].

A limitation of our study is the fact that due to the relatively small study populations, we were not able to make any correlation among clinical symptoms, endoscopic findings, and microscopic features. As only a minority of our patients had symptoms (2 had bleeding that settled without specific intervention) or endoscopic finding of radiation proctitis (4 patients), further studies are needed in order to examine a putative relation between histological findings and clinical symptoms. Moreover, the period that we choose to evaluate our patients could raise concerns, as late radiation-induced rectal injury might occur months or years after radiotherapy. We evaluated our patients with endoscopy 6 months after radiotherapy completion because our objective was to identify factors of prognostic significance regarding the course of the disease. Early proctoscopy, even in asymptomatic but endoscopically confirmed rectal damage, has a significant role in predicting late radiation-induced proctitis [28, 29].

In conclusion, our study showed that in postradiation rectal biopsy specimens neoangiogenesis seems to be inflammation-related and constitutes a significant postradiation component of the tissue injury. The involvement of

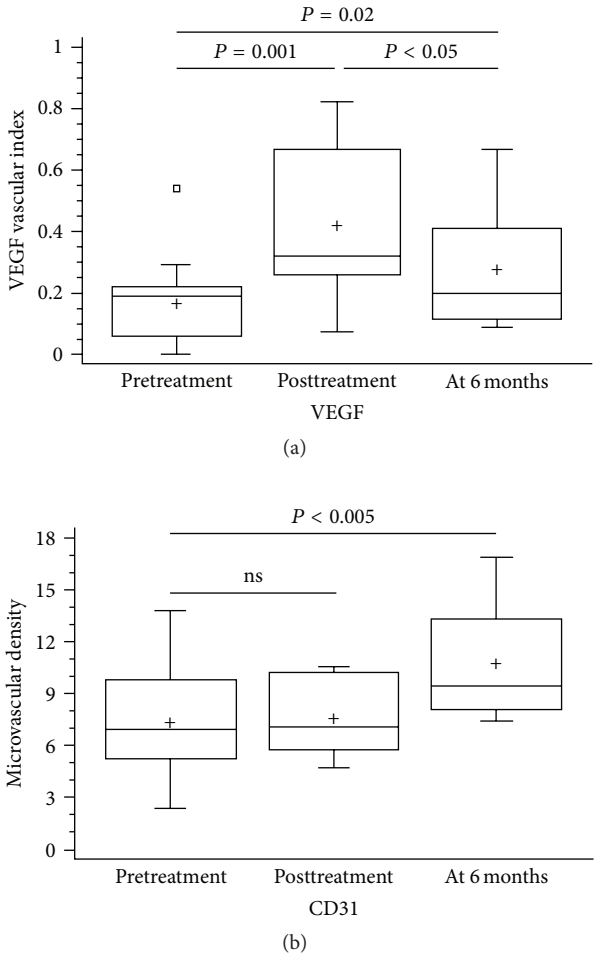


Figure 3: Evaluation of immunostaining for vascular endothelial growth factor (VEGF vascular index) and CD31 (microvascular density) in rectal mucosa of patients with radiation proctitis due to radiotherapy for prostate cancer at three time settings. VEGF vascular index (a) was significantly increased at the completion of irradiation and remained so, at 6 months. In contrary, microvascular density (b) was unchanged at the completion of radiotherapy but increased significantly at 6 months.

inflammation meditator VEGF in the pathogenesis of radiation proctitis suggests that the blockage of the expression of this factor may represent a promising therapeutic option in patients with refractory to available therapies cases of the disorder.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 939047, 8 pages
http://dx.doi.org/10.1155/2013/939047

Clinical Study

Differential IL-13 Production by Small Intestinal Leukocytes in Active Coeliac Disease versus Refractory Coeliac Disease

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Received 2 February 2013; Accepted 4 March 2013

Academic Editor: David Bernardo Ordiz

A small fraction of coeliac disease (CD) patients have persistent villous atrophy despite strict adherence to a gluten-free diet. Some of these refractory CD (RCD) patients develop a clonal expansion of lymphocytes with an aberrant phenotype, referred to as RCD type II (RCDII). Pathogenesis of active CD (ACD) has been shown to be related to gluten-specific immunity whereas the disease is no longer gluten driven in RCD. We therefore hypothesized that the immune response is differentially regulated by cytokines in ACD versus RCDII and investigated mucosal cytokine release after polyclonal stimulation of isolated mucosal lymphocytes. Secretion of the T_H2 cytokine IL-13 was significantly higher in lamina propria leukocytes (LPLs) isolated from RCDII patients as compared to LPL from ACD patients ($P = 0.05$). In patients successfully treated with a gluten-free diet LPL-derived IL-13 production was also higher as compared to ACD patients ($P = 0.02$). IL-13 secretion correlated with other T_H2 as well as T_H1 cytokines but not with IL-10 secretion. Overall, the cytokine production pattern of LPL in RCDII showed more similarities with LPL isolated from GFD patients than from ACD patients. Our data suggest that different immunological processes are involved in RCDII and ACD with a potential role for IL-13.

1. Introduction

Coeliac disease (CD) is an autoimmune enteropathy that is triggered by the gliadin fraction of dietary gluten peptides [1]. The immune processes in CD have been widely studied and it is commonly accepted that in CD innate and adaptive immune responses are part of the pathogenesis [2]. Gliadins can exert direct toxic effects by binding to epithelial cells, resulting in the production of IL-15 and TNFα [3–5]. IL-15 upregulates natural-killer receptors on intra-epithelial cytotoxic T lymphocytes as well as their ligands on epithelial cells, which leads to enhanced apoptotic killing of epithelial cells [6]. The main pathogenic mechanism of CD, however, is believed to be a gluten-specific T_H1-mediated response resulting in an overexpression of IFNγ in the (intra) epithelial compartment [7]. IFNγ, together with TNFα, enhances the expression of transglutaminase-2 (TG2) [8]. TG2 binds and deamidates gliadin peptides, which leads to a better presentation of gliadin peptides to specific T_H cells and a subsequent stronger gliadin-specific immune response with even higher amounts of IFNγ [9, 10]. Although the exact mechanism is unknown, evidence exists that the overexpressed IFNγ ultimately leads to the mucosal damage found in CD [11, 12]. More recently, the proinflammatory cytokine IL-17A has been found to play an important role in coeliac pathology as well [13]. Despite a predominant proinflammatory cytokine profile in active CD, also expression of the regulatory cytokine IL-10 is found, possibly limiting the production of proinflammatory cytokines [14]. Indeed, in a pilot phase I study, treatment with recombinant IL-10 did induce some relief of symptoms in a minority of patients but IL-10 treatment did not lead to mucosal recovery [15].

In contrast to uncomplicated CD, less is known about the pathology of refractory coeliac disease (RCD) [16]. RCD is

a complication of CD in which patients despite following a strict gluten-free diet (GFD) do not recover from symptoms and mucosal lesions. RCD type II (RCDII) is characterized by a significant (>20%) aberrant intraepithelial T lymphocyte (IEL) population in the small intestinal mucosa. These aberrant IEL lack T-cell-specific surface markers, that is, T-cell receptor (TCR), CD3, CD4, and CD8, but express cytoplasmic CD3. Clonal expansion of these aberrant IEL is thought to be responsible for the occurrence of enteropathy-associated T-cell lymphoma (EATL), which occurs in 60%–80% of RCDII patients within 5 years [17]. Similarly to uncomplicated, active CD, IL-15 and IFNγ are reported to be enhanced in RCD; however it is unclear whether they play significant roles in the pathogenesis of RCD [18, 19]. TNFα may play a role in RCD, since some RCD cases have been described where anti-TNFα therapy has shown to have a beneficial effect [20, 21]. IL-17A, IL-13, and IL-5 have not yet been investigated in RCD.

As in RCD the immunological trigger gliadin is absent, we hypothesized that the cytokine profile of IEL and lamina propria leukocytes (LPL) is altered as compared to the gliadin-driven immune response in ACD. Therefore, we measured protein levels of the proinflammatory cytokines TNFα, IFNγ, and IL-17A, the T_H2 cytokines IL-13 and IL-5, and the regulatory cytokine IL-10, in supernatants of polyclonally stimulated leucocytes from biopsies of uncomplicated CD and RCD patients.

2. Patients and Methods

2.1. Patients. Consecutive patients ($n = 20$) were included in our study that visited our outpatient clinic for CD or RCD follow-up. Biopsies were taken for diagnostic purposes and cells remaining from the diagnostic procedure were used for our experiments. The study protocol adhered to the guidelines set by our institutional ethical committee. Patients with concomitant complications such as ulcerative jejunitis or autoimmune enteropathy and patients with collagenous sprue were excluded. Active CD (ACD) was diagnosed according to current guidelines for adult CD [22], that is, if biopsies showed increased numbers of intraepithelial lymphocytes, crypt hyperplasia, and villous atrophy together with antibodies against transglutaminase-2 (TG2A) and endomysium. CD patients were prescribed a gluten-free diet (GFD) and were considered recovered when TG2A levels normalized and when follow-up biopsies showed no villous atrophy anymore (Marsh 0–II; GFD patient group). Adherence to a GFD was confirmed by a dietitian and absence of TG2A in serum. Follow-up biopsies were taken in order to confirm histological recovery or when CD symptoms persisted and RCD was suspected.

Patients were diagnosed with RCD when malabsorption symptoms and histological abnormalities persisted or recurred despite strict dietary adherence (as confirmed by the disappearance of TG2A and EMA) and after exclusion of other intestinal diseases. RCDII was diagnosed, if an aberrant IEL population ($CD3^-$, intracellular $CD3^+$, $CD7^+$) occurred with a frequency of more than 20% of all IEL [23]. Since the distinction between RCDI and slow responders on a GFD can only be done after a long-term follow-up, patients with suspected RCDI were excluded and only patients with RCDII were included in this study. RCDII patients were treated with autologous stem cell transplantation (SCT), 6-thiogunidine (6-TG), cladribine, or entocort; one patient was analysed prior to treatment (Table 2). Similarly to CD patients, RCDII patients were considered recovered, when villous atrophy was absent after therapy.

2.2. Cell Cultures and Cytokine Measurement. Small intestinal biopsies were separated into epithelial layer and lamina propria by incubation in PBS containing DDT and EDTA in a 37°C shaking water bath for one hour as previously described [24]. IEL were washed and collected in ice-cold PBS-BSA 0.1%. The remaining lamina propria was incubated for 2 h in PBS with 10% FCS and 0.16 U/mL collagenase (Collagenase A, Roche). After incubation the biopsies were passed through a sterile 100 μm and filtered through a sterile 40 μm mesh. Cells were then washed and collected in ice-cold PBS containing 0.1% BSA. IEL and LPL were incubated for at least 15 min. with magnetic beads linked to anti-CD45 antibodies (MACS human-CD45 MicroBeads, Miltenyi Biotec). CD45-positive cells (leukocytes) were separated on a magnetic column (MACS MS column, Miltenyi Biotec), collected, and divided over two (IEL) or three (LPL) wells of a 96-well cell-culture plate: IEL: (1) unstimulated, (2) stimulated with 50 ng/mL PMA, 1 μg/mL ionomycin, and 50 ng/mL LPS; LPL: (1) unstimulated, (2) stimulated with 50 ng/mL PMA and 1 μg/mL ionomycin, and (3) stimulated with 50 ng/mL LPS. Each well contained the cells of approximately 2 biopsies in a total volume of 100 μL. After 24 hour incubation at 37°C and 5% CO_2, supernatants were collected, frozen, and stored at −20°C until analysed. Cytokine levels of TNFα, IL-17A, IL-13, IL-10, and IL-5 were determined using a multiplex bead assay (Cytometric Bead Assay, BD). IFNγ was measured using a commercially available ELISA kit (PeliKine compact human IFNγ, Sanguin).

2.3. FACS Analyses. Cell subsets, that is, $CD4^+$ and $CD8^+$ T cells, CD3-$CD16/56^+$ NK cells, and $CD19^+$ B-cells, were determined by multicolour FACS analysis using CD3-FITC, CD8-PE, CD45-PerCP, and CD4-APC and CD3-FITC, CD16/56-PE CD45-PerCP, and CD19-APC antibody conjugates, respectively (Multitest, BD). Aberrant IEL were analysed by surface CD3, CD52, and CD45 followed by cytoplasmic staining of CD3 after cell permeabilization (Cytofix/CytoPerm Plus kit, BD Biosciences). All analyses were performed on lymphocytes, based on bright CD45 staining and low side scatter (SSC). Aberrant T cells were defined as $CD52^+$ cytoplasmic $CD3^{++}$ and surface CD3 negative cells. Total numbers of IEL (cell harvest) were determined using FACS tubes containing a fixed number of reference beads (Trucount tubes, BD).

2.4. Statistical Analyses. Differences in cytokine levels were tested with the Mann-Witney U test. Difference in sex distribution was tested with the chi-square test. Differences

Table 1: Patient characteristics and composition of leucocyte infiltrates.

	ACD $N = 4$	GFD $N = 7$	RCDII $N = 9$
Sex, % females	75.0%	71.4%	33.3%
Age, yrs	45.8 (22.2–75.3)	55.9 (35.3–72.0)	70.6 (41.7–76.2)
Villous atrophy, %	100%	0.0%	45.5%
Cell yield, 10^3 IEL/biopsy	28.5 (7.5–58.0)	16.0 (2.1–113.0)	19.1 (5.8–62.4)
$CD3^+$ IEL, % of CD45	99 (97–99)	96 (86–98)	21 (10–99)*
$CD4^+$ IEL, % of CD45	5 (1–10)	3 (2–32)	5 (1–14)
$CD8^+$ IEL, % of CD45	77 (59–86)	78 (65–90)	13 (5–69)*
$CD16/56^+$ IEL, % of CD45	1 (0–3)	2 (1–11)	3 (0–24)
Aberr. IEL, % of CD45	0 (0-1)	2 (0–6)	66 (1–87)*
$CD3^+$ LPL, % of CD45	40–43	21–60	25–38
$CD4^+$ LPL, % of CD45	13–28	0–31	8–24
$CD8^+$ LPL, % of CD45	10–16	2–29	4–14
$CD16/56^+$ LPL, % of CD45	3-3	5–8	1–4
$CD19^+$ LPL, % of CD45	4-5	1–8	3–10

For age and IEL data medians (5 percentile–95 percentile) are shown. For LPL data ranges are shown, since data for composition of LPL was available only in 2 ACD patients, 4 GFD patients, and 4 RCDII patients.
*Significantly lower percentage of $CD3^+$ and $CD8^+$ cells compared to ACD and GFD (due to high percentage of aberrant T-cells).

Table 2: Patient characteristics of RCDII patients.

	Sex	Age, yrs	Marsh	Treatment	Last treatment < 6 weeks before biopsy	Aberrant cells, % of CD45	Symbol Figures 1 and 2[d]
1[a]	M	68.1	IIIa	Chemotherapy, entocort	Yes	77%	●
2[c]	M	76.0	IIIa	2x cladribine	Yes	37%	■
3	F	72.8	IIIa	Cladribine	No	70%	▲
4	F	41.7	IIIb	None	No	87%	▼
5[b]	F	54.9	IIIc	6-TG	Yes	0.6%	♦
6	M	70.3	I	Cladribine, SCT	No	13%	○
7[c]	M	76.2	0	Cladribine	Yes	41%	□
8	M	72.9	I	SCT	No	73%	△
9	M	70.6	I	Cladribine	No	66%	◊

[a]RCDII after successful treatment of enteropathy-associated T cell lymphoma.
[b]Enteropathy-associated T cell lymphoma was diagnosed when biopsy was taken.
[c]Patients 2 and 7 are the same patients before and after histological recovery.
6-TG: 6-thioguanine, SCT: stem cell transplantation.
[d]Corresponding symbol in Figures 1 and 2.

in age, cell count, and cell type ratios were tested with the student's t-test. Correlation coefficients were calculated with a two-sided Pearson's correlation.

3. Results

3.1. Patient Characteristics and Composition of Leukocyte Infiltrates. A total of 20 patients were included in our study: 4 patients with active coeliac disease (ACD), 7 on a gluten-free diet (GFD), and 9 patients with RCDII. RCDII patients tended to be older at the time of cytokine analysis than ACD patients ($P = 0.07$, Table 1). The follow-up time of GFD patients was at least 8 months, and that of RCDII patients at least 2 years since the start of the gluten-free diet (data not shown).

Five of the RCDII patients had villous atrophy. One of these patients was not treated and four retained villous atrophy despite treatment (Table 2). Of the four patients that recovered histologically after treatment, one was treated with SCT, and the other three with cladribine.

Cell yield (total number of isolated IEL) did not differ significantly between groups. The median cell yield was highest in ACD patients with 28,500 cells per biopsy compared to GFD (16,000 cells per biopsy) and RCDII (19,100 cells per biopsy). Due to large variation, however, no significant difference in cell yield was observed between groups. The percentage of CD3-positive IEL, mostly $CD8^+$ T-cells, was significantly lower in RCDII patients compared to GFD and ACD, which is due to the high percentage of aberrant IEL found in RCDII patients (Table 1). NK cell frequencies in IEL

were generally low (Table 1) and B-cells were absent (data not shown). In the LPL fraction NK cell and B-cell frequencies were below 10% in all groups (Table 1).

3.2. Cytokine Levels in IEL. Stimulation of IEL overall resulted in low cytokine levels, probably due to the generally low numbers of leukocytes present in the epithelial layer. Only IFNγ and TNFα, both known to be increased in the duodenum of CD patients, reached detectable levels in IEL. In order to analyse whether IEL numbers may influence possible differences in cytokine levels between the groups, the amount of cytokine was divided by the number of IEL that were isolated from biopsies. No significant differences could be found between ACD and RCDII patients whether the amount of cytokine per 1000 IEL (Figures 1(a) and 1(b)) or the amount of cytokine per two biopsies (Figures 1(c) and 1(d)) was analysed. IFNγ production was not lower in GFD patients as compared to ACD patients. However, in the RCDII group, IEL-derived IFNγ production was the highest in patients with persisting villous atrophy (Figures 1(a) and 1(b), closed symbols).

3.3. Cytokine Levels in LPL. LPLs were stimulated with either PMA/ionomycin to trigger all the leukocytes or LPS to trigger antigen-presenting cells (APC) only. After LPS stimulation most cytokines were undetectable and only low levels of IFNγ and TNFα were detectable in a minority of the patients. For both IFNγ and TNFα no differences could be observed between groups after LPS stimulation (data not shown). As in general the levels were 50- to 500- fold lower after LPS stimulation as compared to PMA/ionomycin stimulation, IFNγ and TNFα production after PMA/ionomycin will be mostly lymphocyte rather than APC derived.

In contrast to LPS, stimulation of LPL with PMA/ionomycin resulted in detectable cytokine levels. RCDII patients who were treated within 6 weeks before the biopsy was taken appeared not to be different in terms of cytokine production from patients who were treated more than 6 weeks before the biopsy was taken (Figure 2 and Table 2). However, levels of most cytokines (IFNγ, TNFα, IL-13, and IL-17A) tended to be the highest in patients with persisting villous atrophy (Figure 2, closed symbols). Similar to the IEL results, IFNγ production by LPL was comparable between ACD and RCDII patients and IFNγ production was not reduced in GFD patients compared to ACD (Figure 2(a)). IL-13 responses were higher in RCDII when compared to ACD patients but were also higher in GFD as compared to ACD (Figure 2(c)). Since IL-13 production was significantly increased in RCDII patients as compared to ACD patients, we analysed the coexpression of IL-13 and the other cytokines by calculating correlation coefficients for all IL-13 cytokine pairs. IL-13 release correlated the strongest with IL-17A and TNF ($r = 0.80$ and $r = 0.73$, resp.; both $P < 0.001$; Figures 3(a) and 3(b)). Weaker correlations were observed with IL-5 and IFNγ ($r = 0.63$, $P = 0.003$ and $r = 0.45$, $P = 0.04$, resp.; Figures 3(c) and 3(d)), while there was no significant correlation between IL-13 and IL-10 ($r = 0.38$, $P = 0.10$; Figure 3(e)).

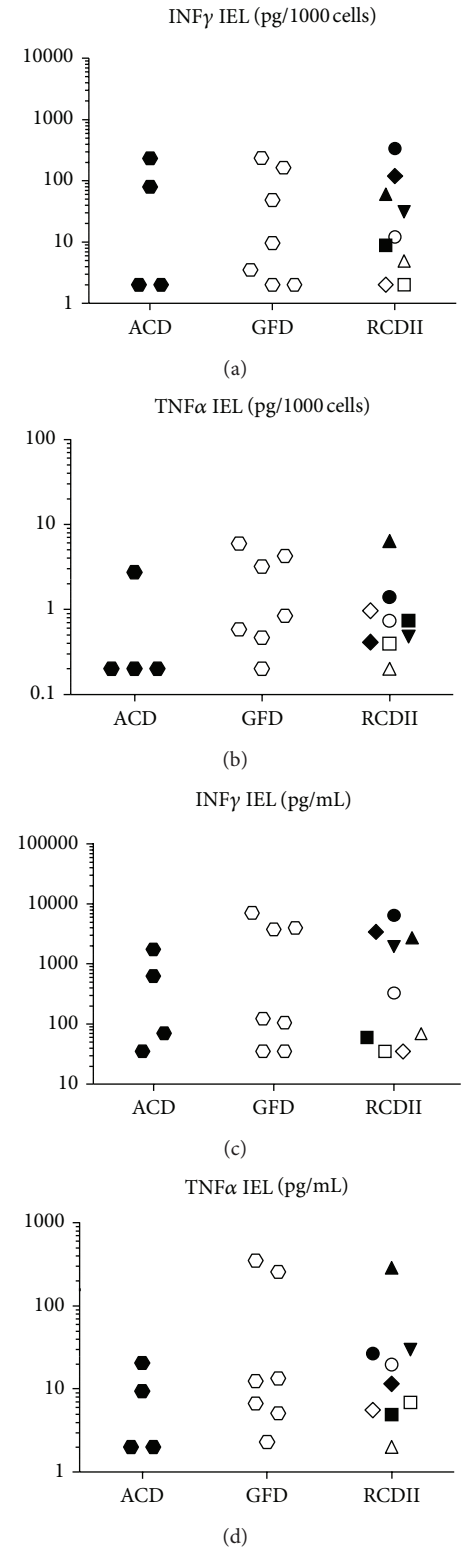

Figure 1: Production of INFγ and TNFα by IEL from active CD patients (ACD), patients on a gluten-free diet (GFD), and refractory CD type II (RCDII) patients after PMA/ionomycin/LPS stimulation. RCDII patients with villous atrophy (closed symbols); RCDII patients without villous atrophy (open symbols); for individual characteristics see Table 2. ((a), (c)) IFNγ and ((b), (d)) TNFα production. ((a), (b)) Production per 1000 IEL or ((c), (d)) per mL per two biopsies.

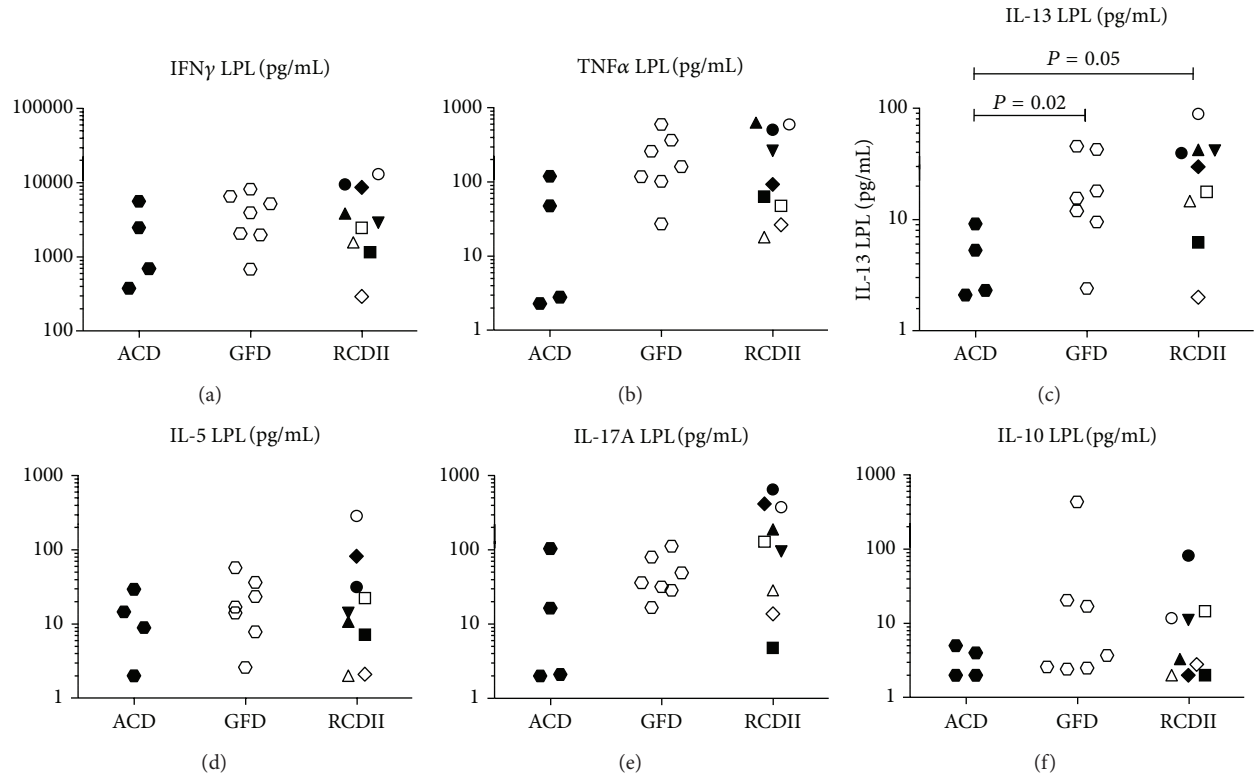

Figure 2: Production of (a) IFNγ, (b) TNFα, (c) IL-13, (d) IL-5, (e) IL-17, and (f) IL-10 by LPL from active CD patients (ACD), patients on a gluten-free diet (GFD), and refractory CD type II (RCDII) patients after PMA/ionomycin stimulation. RCDII patients with villous atrophy (closed symbols); RCDII patients without villous atrophy (open symbols). Groups were compared using the Mann-Whitney U test. P values are shown for significant differences.

4. Discussion

In this study we tested the hypothesis that the local cytokine profile would be different in gluten-driven ACD as compared to gluten-independent RCDII. This was investigated by analysing the capacity of LPL and IEL isolated from the duodenum of ACD and RCDII patients as well as from patients successfully treated with a gluten-free diet to produce IFNγ, TNFα, IL-17A, IL-13, IL-5, and IL-10. IL-15 was not analysed as it is not well secreted and unstable [25].

IFNγ production has been extensively studied in ACD and GFD. While IFNγ has been considered to play an important role in enterocyte destruction in ACD, several studies have shown that IFNγ levels are not reduced in GFD [26, 27]. This is in line with our findings that show no difference in the capacity to produce IFNγ between IEL/LPL from ACD and GFD patients. Here, we also show that there is no increase in IFNγ production in RCDII patients. This suggests that the capacity of IEL/LPL to produce IFNγ appears not to be solely dependent on an ongoing gluten-driven immune response.

In contrast to our findings here, levels of TNFα protein have been found to be elevated in lamina propria and epithelium of ACD patients and decreased after a GFD [28, 29]. However, there are important methodological differences between the present and these previous studies. While we used PMA and ionomycin stimulation to analyse the capacity of the IEL/LPL to produce particular cytokines, the above mentioned studies used RT-PCR analysis or immunohistochemistry to analyse cytokine mRNA levels or protein without prior stimulation. This suggests that the capacity of IEL and LPL to produce TNFα may be similar in ACD and GFD while the current production at the time of biopsy may be reduced in GFD.

Although there is a considerable overlap between the groups, the capacity of LPL to produce IL-13 and IL-17A seems to be lower in ACD as compared to RCDII and GFD, which reached statistical significance for IL-13 when analysed individually. In paediatric ACD patients, lower numbers of mucosal T cells with the capacity to produce IL-17A were observed as compared to controls. It was suggested that the relative lack of IL-17A producing T cells may affect the homeostasis of the epithelial layer and contribute to increased intestinal permeability [30]. In our dataset this was less apparent; however, in a subset of RCDII patients (particularly those with persistent villous atrophy despite treatment) high levels of IL-17A were detected after polyclonal stimulation and in only one of the ACD patients, suggesting a differentially driven IL-17A response in treatment-resistant RCDII patients. This increased capacity of LPL to produce IL-17A in treatment-resistant RCDII may be related to the continued inflammation and risk of EATL development, as IL-17A is involved in chronic inflammation as well as in tumour formation [31].

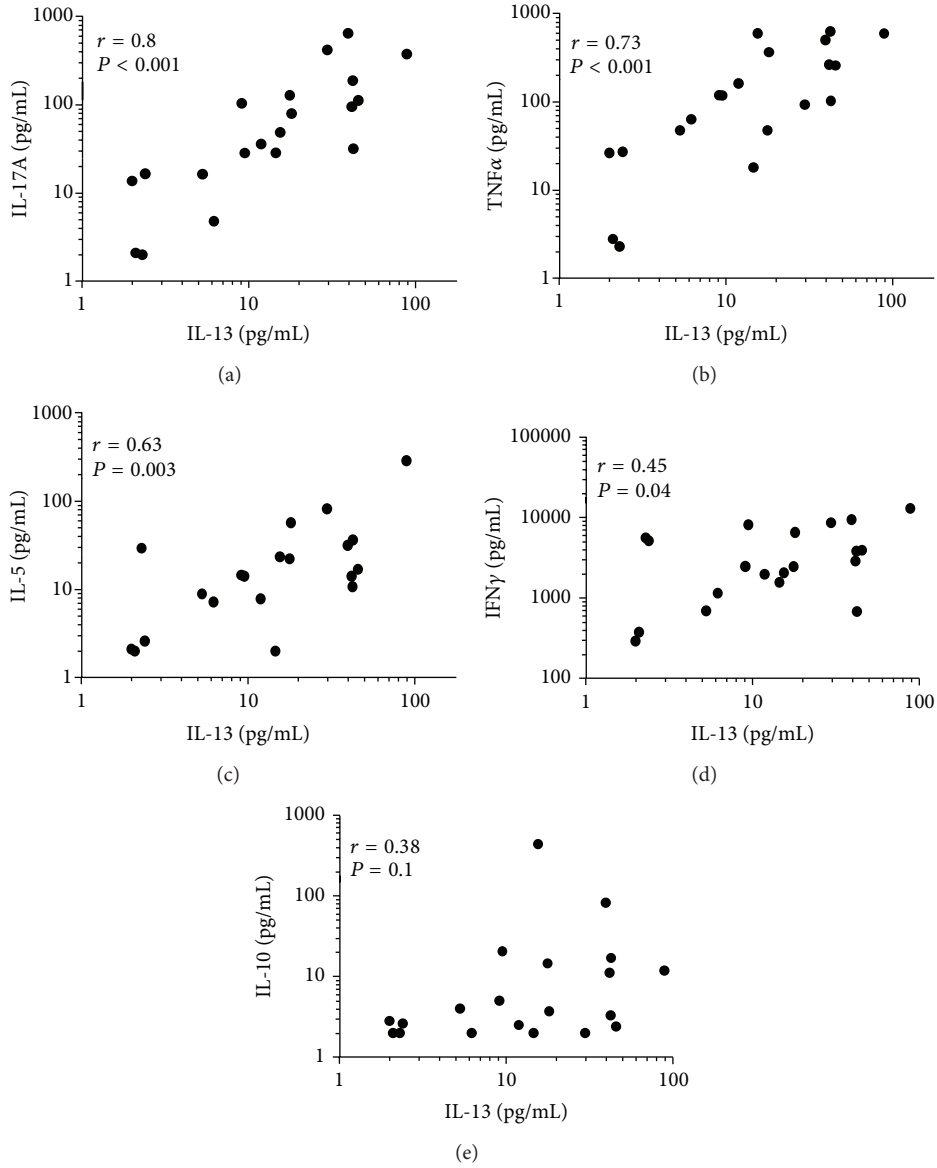

Figure 3: Correlation between (a) IL-13 and IL-17A, (b) TNFα, (c) IL-5, (d) IFNγ and (e) IL-10 production in all groups. Correlations were tested with a two-sided Pearson correlation.

To the best of our knowledge this is the first study that investigated local IL-13 levels in CD and RCD. In our experiments we found higher IL-13 production in RCDII patients as compared to ACD patients. IL-13 production capacity was also higher in GFD patients compared to ACD. Although IL-13 is mainly associated with airway pathology, it also has an important role in gut defence and inflammation [32]. In ulcerative colitis the high levels of IL-13 are shown to be derived from variant CD1d-restricted NKT cells and IL-13 has been shown to have a toxic effect on colonic epithelial cells [33, 34]. IL-13 has also been shown to be produced by NK cells as part of an innate response [35]. This is in line with the high levels of IL-13 found in RCDII where antigenic stimulation by gluten is lacking. The higher IL-13 production was not related to NK cell frequencies; whether the IL-13 produced is NK or variant NKT cell derived remains to be investigated. IL-13 production capacity was not only correlated to IL-17A production but also to the other T_H1 and T_H2 cytokines, but not to the regulatory cytokine IL-10, which is in line with a proinflammatory role for this cytokine.

IL-13 has been shown to have direct cytotoxic effects on epithelial cells. It is, therefore, intriguing to speculate why there is an increased capacity of LPL to produce IL-13 in both patients on a successful GFD and RCDII patients. Differential expression of the receptors on epithelial cells as has been described for the IL-15 receptor [36] as well as regulatory cytokines not measured here (TGFβ) or contact-dependent regulation by regulatory cells may play a role. Although the difference between RCDII and ACD was only statistically significant for the IL-13 production capacity, the production

pattern of the other cytokines was comparable, and the overall cytokine profile of LPL in RCDII showed more similarities with LPL from GFD patients than from ACD patients.

It has to be taken into account that for this study we did not have healthy controls available to compare our results to. It is therefore unclear whether GFD patients and RCD patients had increased IL-13 levels or ACD had reduced IL-13 levels.

5. Conclusions

In conclusion our data show that IL-13 production is lower in the lamina propria of ACD patients, compared to GFD and in particular RCDII patients, suggesting that the immune responses in ACD and RCDII are differently regulated and that IL-13 may play a role as a proinflammatory cytokine in the pathogenesis of RCDII.

Abbreviations

6-TG: 6-thioguanine
ACD: Active coeliac disease
RCD: Refractory coeliac disease
RCDII: Refractory coeliac disease type II
DDT: Dithio-DL-threitol
EDTA: Ethylen-diamin-tetra-acetic acid
GFD: Gluten-free diet
IL: Interleukin
IFNγ: Interferon gamma
LPS: Lipopolysaccharide
PMA: Phorbol myristate acetate
SCT: Stem cell transplantation
T_H: T helper
TG2A: Transglutaminase-2 (= tissue transglutaminase) antibodies
TNFα: Tumor necrosis factor alpha.

Conflict of Interests

The authors do not have any conflict of interests to disclose.

Acknowledgments

The authors would like to thank Jolien Hollander and Martine Reijm for excellent technical assistance. This study was funded by the Coeliac Disease Consortium (CDC round 2; NGI 05060451).

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 684237, 10 pages
http://dx.doi.org/10.1155/2013/684237

Clinical Study

Expression of Inflammation-Related Genes Is Altered in Gastric Tissue of Patients with Advanced Stages of NAFLD

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Received 15 December 2012; Revised 12 February 2013; Accepted 14 February 2013

Academic Editor: David Bernardo Ordiz

Obesity is associated with chronic low-grade inflammation perpetuated by visceral adipose. Other organs, particularly stomach and intestine, may also overproduce proinflammatory molecules. We examined the gene expression patterns in gastric tissue of morbidly obese patients with nonalcoholic fatty liver disease (NAFLD) and compared the changes in gene expression in different histological forms of NAFLD. Stomach tissue samples from 20 morbidly obese NAFLD patients who were undergoing sleeve gastrectomy were profiled using qPCR for 84 genes encoding inflammatory cytokines, chemokines, their receptors, and other components of inflammatory cascades. Interleukin 8 receptor-beta (*IL8RB*) gene overexpression in gastric tissue was correlated with the presence of hepatic steatosis, hepatic fibrosis, and histologic diagnosis of nonalcoholic steatohepatitis (NASH). Expression levels of soluble interleukin 1 receptor antagonist (*IL1RN*) were correlated with the presence of NASH and hepatic fibrosis. mRNA levels of interleukin 8 (*IL8*), chemokine (C-C motif) ligand 4 (*CCL4*), and its receptor chemokine (C-C motif) receptor type 5 (*CCR5*) showed a significant increase in patients with advanced hepatic inflammation and were correlated with the severity of the hepatic inflammation. The results of our study suggest that changes in expression patterns for inflammatory molecule encoding genes within gastric tissue may contribute to the pathogenesis of obesity-related NAFLD.

1. Background

Obesity is a multisystem disorder characterized by an excessive increase in the adipose tissue. Biochemically, obesity can be defined as a failure of the normal energy homeostasis mechanisms which are required to balance the intake and the expenditure of energy [1, 2]. The regulation of the size of fat stores is a complex process and involves both central and peripheral tissues [1, 3] and over 50 secreted molecules, such as the adipocytic hormones leptin and adiponectin [4, 5], gastric ghrelin [6, 7], and intestinal cholecystokinin [8]. Many of these molecules also play a role in various diseases associated with obesity, particularly, nonalcoholic fatty liver disease (NAFLD) [7, 9].

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of diseases ranging from relatively benign fatty liver (simple steatosis) to nonalcoholic steatohepatitis, or NASH, characterized by inflammation and ballooning degeneration of hepatocytes, which may progress to fibrosis or cirrhosis. NAFLD is considered to be the hepatic manifestation of metabolic syndrome affecting both adults and children [10, 11] and is thought to reach a prevalence of up to 30% in the general population [11–13]. The association of NAFLD with obesity, particularly visceral obesity, has long been recognized [12]. Although a number of pathways, such as enhanced oxidative stress, increased susceptibility to apoptosis, and insulin resistance have been implicated in the pathogenesis of NAFLD [11], little is known about the triggers of the progression

to NASH, hepatic fibrosis, and ultimately cirrhosis. Not all individuals with NAFLD progress to cirrhosis. Additionally, not all obese patients develop NASH. One explanation for this differential progression maybe the contribution of nonadipose peripheral tissues to the pathogenesis of obesity-related NAFLD. Given that the stomach is one of the central organs of the digestive tract relaying satiety signals to the hypothalamus [14, 15] and is a source of peptides with critical roles in energy homeostasis (ghrelin), its participation in the development of obesity related NAFLD or its progression looks plausible. The discovery of ghrelin and its role in human metabolism has intensified the studies of hypothalamic control of the appetite and its contribution to obesity [16]. In 2005, it was found that the ghrelin-encoding gene also encodes obestatin, which, unlike ghrelin, is involved in appetite suppression [17]. In addition to ghrelin and obestatin, the stomach is the second largest source, after adipose tissue, of the appetite inhibiting peptide leptin [18–20]. Yet, studies on the role of gastric tissue in obesity-related disorders, such as NAFLD, are scarce.

In our previous study, we showed that the serum levels for common stomach hormones are altered in patients with advanced stages of NAFLD [7]. In particular, concentrations of des-acylghrelin in serum of patients with NASH were increased twofold as compared to BMI-matched controls with simple steatosis, while concentrations of ghrelin and obestatin were increased in patients with advanced liver fibrosis [7]. Other studies showed that the levels of ghrelin are related to inflammation and reduce the severity of inflammation [21, 22]. An overproduction of the ghrelin in the patients with advanced stages of chronic liver disease may be a compensatory event or a reflection of local inflammatory responses on site of their production.

Observations listed above prompted us to hypothesize that the gastric tissue in obese subjects is actively contributing to the systemic inflammation and pathogenesis of one of the complications of obesity, NAFLD. To investigate this, we performed comparative expression profiling for 84 genes encoding inflammatory cytokines, chemokines, their receptors and other components of inflammatory cascades in samples of gastric tissue removed during sleeve gastrectomy.

2. Methods

2.1. Samples. This study was approved by Inova Institutional Review Board (Federal Assurance FWA00000573). After informed consent, 20 morbidly obese NAFLD patients undergoing laparoscopic sleeve gastrectomy were included. For each patient, a large number of clinical and laboratory variables were available. Other chronic liver diseases were excluded by negative serology for hepatitis B and C, no history of toxic exposure and no other cause of chronic liver disease. Excessive alcohol consumption (>10 grams/day in women and >20 grams/day in men) was also excluded. No patients were receiving thiazolidinediones (TZDs) or medications for gastritis, including proton pump inhibitors.

From each patient, a discarded gastric tissue during sleeve gastrectomy was obtained and snap frozen with liquid nitrogen. Every gastric sample was also evaluated histologically for the presence of gastritis. As noted, samples were flash frozen in liquid nitrogen, placed in −80°C. Gene expression profiling experiments were performed using fundic samples collected from the remaining sleeve gastrectomy specimens. Samples were profiled for expression levels of 84 genes encoding inflammatory cytokines, chemokines, their receptors, and other components of inflammatory cascades using RT^2 Profiler PCR Arrays (Qiagen, USA) (see Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/684237).

For each patient, a liver biopsy was performed and read by the hepatopathologist. Before histopathological evaluation, each liver biopsy specimen was formalin-fixed, sectioned, and stained with hematoxylin-eosin and Masson's trichrome. The slides were reviewed following a predetermined histologic grading system; the extent of steatosis was graded as an estimate of the percentage of tissue occupied by fat vacuoles as follows: 0 = none, 1 ≤ 5%, 2 = 6–33%, 3 = 34–66%, and 4 ≥ 66%. Other histological features evaluated in H & E sections included portal inflammation, lymphoplasmacytic lobular inflammation, polymorphonuclear lobular inflammation, Kupffer cell hypertrophy, apoptotic bodies, focal parenchymal necrosis, glycogen nuclei, hepatocellular ballooning, and Mallory-Denk bodies. Patients who had hepatic steatosis (with or without nonspecific inflammation) or NASH were considered to have NAFLD. NASH was defined as steatosis, lobular inflammation, and ballooning degeneration with or without Mallory-Denk bodies and with or without fibrosis. Hepatic inflammation was defined according to an extent of immune cell infiltration (lymphoplasmacytic cells, polymorphonuclear cells, and Kupffer cell hypertrophy). For each category, score was assigned based on the following system: 0 = none, 1 = few, 2 = moderate, and 3 = many. Severity of total hepatic inflammation was determined based on the sum of the individual scores with advanced hepatic inflammation ≥3 and mild/no hepatic inflammation <3. Severity of pericellular and portal fibrosis was determined based on a similar scoring system as follows: 0 = none, 1 = mild, 2 = moderate, and 3 = marked fibrosis. Severity of total hepatic fibrosis was determined based on the sum of the individual scores (pericellular and portal fibrosis) with a score of ≥3 being considered as advanced hepatic fibrosis and a score of <3 being considered as mild/no hepatic fibrosis.

2.2. RNA Extraction and Reverse Transcription. Total RNA was extracted from fundic gastric tissue samples ($N = 20$) using RNeasy kit (Qiagen, USA) according to manufacturer's instructions. To determine the quantity and purity of the extracted RNA, absorbances were measured at 260 nm (A260) and 280 nm (A280) by the GeneQuant1300 spectrophotometer (GE Healthcare, USA). RNA of A260/A280 ratio of 1.8–2.1 was considered of high purity. RNA integrity was confirmed by gel electrophoresis using 1% agarose with ethidium bromide. RNA with sharp, clear 28S and 18S ribosomal RNA (rRNA) bands and the intensity of 28S rRNA band approximately twice as intense as the 18S rRNA band were used as parameters to evaluate the integrity of total RNA. 560 ng of extracted total RNA was reverse transcribed

Table 1: Demographic and clinical characteristics of the patient cohorts profiled for expression of inflammation- and immunity-related genes, values marked by asterisk (∗) are given as average ± SD. All subjects qualified for NAFLD had no history of alcohol abuse. No patients were taking thiazolidinediones (TZDs) or medication for gastritis.

Demographic or clinical parameter	Mean ± SD, or % ($N = 20$)
BMI (∗)	48.67 ± 8.95
AST, U/L (∗)	24.20 ± 7.13
ALT, U/L (∗)	31.25 ± 12.99
Total cholesterol, mg/dL (∗)	185.40 ± 76.61
HDL, mg/dL (∗) females	53 ± 17
HDL, mg/dL (∗) males	39.67 ± 7.09
Triglyceride, mg/dL (∗)	196.75 ± 107.28
Glucose, mg/dL (∗)	108.70 ± 36.20
Age, yr	43.44 ± 10.63
Hypertension	55% ($N = 11$)
Smoking	5% ($N = 1$)
Gender (females)	75% ($N = 15$)
Race (Caucasian)	80% ($N = 16$)
Advanced inflammation (score ≥ 3)	50% ($N = 10$)
NASH	65% ($N = 13$)
Advanced steatosis	60% ($N = 8$)
Fibrosis	75% ($N = 15$)
Steatosis with advanced inflammation	50% ($N = 10$)
NASH with advanced inflammation	30% ($N = 6$)
Gastritis	45% ($N = 9$)

SD: standard deviation; BMI: body mass index; NASH: nonalcoholic steatohepatitis; AST: aspartate aminotransferase; ALT: alanine transaminase; HDL: high-density lipoprotein.

using RT^2 first strand kit (Qiagen, USA). According to manufacturer's protocol, total RNA was treated to eliminate genomic DNA. Both random hexamers and oligo-dT primers were used to prime reverse transcription performed as recommended by enzyme manufacturer (Qiagen, USA).

2.3. Quantitative Real Time PCR Analysis. Quantitative real-time PCR was performed in 96 well PCR format using Bio-Rad CFX96 Real Time System (BioRad Laboratories, USA) with a ramp speed of 1°C/sec. Inflammatory cytokines and receptor RT^2 Profiler PCR Arrays (Qiagen, USA) were used to simultaneously examine the mRNA levels of 84 genes encoding for inflammatory cytokines, their receptors and intracellular components of inflammatory cascades along with five housekeeping genes following the manufacturer's protocol. The real-time PCR mixtures consisted of 1 μL cDNA and 7.5 μL of RT PCR master mix (Qiagen, USA) in a final volume of 25 μL. The thermal profile of the RT-PCR procedure was repeated for 50 cycles: (1) 95°C for 10 min; (2) 10 s denaturation at 95°C and 15 s annealing at 60°C (amplification data collected at the end of each amplification step); (3) dissociation curve consisting of 10 s incubation at 95°C, 5 s incubation at 65°C, and a ramp up to 95°C (Bio-Rad CFX96 Real Time System, USA). Melt curves were used to validate product specificity.

The results of the RT^2 Profiler PCR Array were further confirmed by independent qPCR experiments. For the genes with significantly altered expression levels, the primers were designed using Primer3 from NCBI ([23] (Supplementary Table 2). The validation was carried out using the thermal profile for 40 cycles: (1) 95°C for 10 min; (2) 10 s denaturation at 95°C and 15 s annealing at 60°C (amplification data collected at the end of each amplification step); (3) dissociation curve consisting of 10 s incubation at 95°C, 5 s incubation at 65°C, and a ramp up to 95°C (Bio-Rad CFX96 Real Time System, USA). The real-time PCR mixtures consisted of 1 μL cDNA, 5 μL of SsoFast EvaGreen Supermix (Bio-Rad, USA), and 250 nM final concentration of primers (Invitrogen, USA) in a final volume of 10 μL.

2.4. Analysis of Gene Expression Profiles. The gene expression data were presented as relative gene expression data [24]. Values were collected for the threshold cycle (C_t) for each gene, and only C_t values less than 40 were considered for further analysis. Normalization of each target gene was carried out relative to five housekeeping genes [24, 25] according to the manufacturer's instructions (Qiagen, USA). Average of C_t values for five housekeeping genes ($C_t^{\mathrm{AVG\,HKG}}$) on the same array (*B2M, HPRT1, RPL13A, GAPD,* and *ACTB*) was calculated. The normalized ΔC_t was log transformed; resultant values were utilized for calculation of the fold change of each target gene in different cohorts. For each target gene, the fold change was used to compare the gene expression levels in two different groups within a cohort (group A and group B). In this study, group A may be the diseased state and group B the nondiseased state; group A may be the advanced diseased state and group B the mild/nondiseased state.

C_t values of control wells (genomic DNA control, reverse transcriptase control, and positive PCR control) were examined separately for assessing the quality of each run and interpolate variability. For the validation of the PCR array results, we carried out the normalization procedure using previously validated housekeeping genes [26]. The relative gene expression values were calculated as described above.

2.5. Statistical Analysis. This study aimed for uncovering changes in gene expression in the stomach of patients with more advanced forms of NAFLD as compared to these with less advanced forms. Comparisons were performed for the following paired cohorts:

(1) mild or no hepatic inflammation versus advanced hepatic inflammation;

(2) mild steatosis versus advanced steatosis;

(3) histologic NASH versus NAFLD without histologic NASH;

(4) hepatic fibrosis versus NAFLD without hepatic fibrosis.

To assess the significance of gene expression differences between compared groups, univariate analyses were performed using the nonparametric Mann-Whitney test. To

Table 2: List of genes significantly upregulated in gastric tissues of patients with the following pathological conditions.

Genes	Fold change	P values	FDR significance (B-H pass test)
Advanced liver inflammation (score ≥ 3)/mild or no liver inflammation (score < 3)			
CCL4	2.32	0.037635	Yes
CXCL2	2.82	0.031209	Yes
CCR5	3.16	0.025748	Yes
IFNA2	3.91	0.028306	Yes
IL19	3.48	0.025748	Yes
IL1F8	4.03	0.029948	Yes
CXCL6	4.3	0.037635	Yes
IL8	4.82	0.025748	Yes
Advanced steatosis (score ≥ 3)/mild or no steatosis (score < 3)			
IL8RB	1.56	0.027891	Yes
CXCL14	1.77	0.033865	Yes
IL1F10	2.24	0.049141	Yes
NASH/No NASH[‡]			
CCR9	2.96	0.047583	Yes
CCR3	3.44	0.047583	Yes
IL1RN	3.95	0.021559	Yes
IL9	9.49	0.021363	Yes
IL8RA	15.04	0.040682	Yes
Fibrosis presence/no fibrosis[‡]			
CCL17	4.81	0.029	Yes

Advanced liver inflammation (score ≥ 3) ($N = 10$), advanced steatosis (score ≥ 3) ($N = 8$), NASH[‡] ($N = 13$), fibrosis ($N = 15$). [‡]Comparison was performed for groups of patients without the condition listed. NASH: nonalcoholic steatohepatitis.

determine whether two variables covary, and to measure the strength of any relationship, Spearman's coefficient of correlation was used. The independent effect of significant variables ($P \leq 0.05$) on advanced inflammation, NASH, and steatosis was assessed using multiple stepwise regression analysis with both the backward and forward stepwise selection procedures. The multiple test corrections were carried out using Benjamini-Hochberg-Yekutieli procedure that controls the false discovery rate under positive dependence assumptions reflecting known phenomenon of cocorrelation of expression levels for genes involved in the same cellular or organismal process. In case the positive dependent assumption would turn incorrect, assumption-free Benjamini-Hochberg procedure was also applied. Both procedures were executed using Bioconductor. To put our finding into perspective, both Benjamini-Hochberg-Yekutieli approved *P*values and the results of Benjamini-Hochberg test were reported.

3. Results

Clinical and demographic data summarized in Table 1. All the patients were obese with histologically proven NAFLD.

3.1. Gene Expression Differences between Patients with Mild and Advanced Hepatic Inflammation. When cohorts with mild (score < 3) and advanced hepatic inflammation (score ≥ 3) were compared, expression levels for chemokine (C-C motif) ligand 4 (*CCL4*), chemokine (C-C motif) receptor 5 (*CCR5*), chemokine (C-X-C motif) ligand 2 (*CXCL2*), chemokine (C-X-C motif) ligand 6 (*CXCL6*), interferon α2 (*IFNA2*), interleukin 19 (*IL19*), interleukin-1 family member 8 (*IL1F8*), and interleukin 8 (*IL8*), were significantly increased ($P \leq 0.05$) (Table 2). Among these cytokines, *CCL4, CCR5, IFNA2, IL1F8,* and *IL8* were also independently and significantly correlated with hepatic inflammatory scores ($P \leq 0.05$) (Table 3). Chemokine (C-C motif) ligand 21 (*CCL21*) and chemokine (C-C motif) ligand 3 (*CCL3*), on the other hand, were found to be significantly correlated ($P \leq 0.05$) with hepatic inflammatory scores, but did not show significant differential expression in the group-wise comparisons ($P \geq 0.05$) (Table 3).

3.2. Gene Expression Differences between Patients with Advanced Hepatic Steatosis and Mild or No Hepatic Steatosis. In patients with advanced hepatic steatosis (score ≥ 3), chemokine (C-X-C motif) ligand 14 (*CXCL14*), interleukin-1 family member 10 (*IL1F10*), and interleukin 8 receptor β (*IL8RB*) had a significant differential expression ($P \leq 0.05$) as compared to those with mild steatosis (score ≤ 2) (Table 2). In addition, *IL8RB* and *IL1F10* levels were positively correlated with a degree of steatosis ($P \leq 0.05$) (Table 3).

3.3. Gene Expression Differences between Patients with NASH and without NASH. Patients with presence of histologic

Table 3: Correlations between inflammatory gene expression levels (dependent variable) and the following pathological conditions (independent variable).

Gene	Spearman correlation	P values	FDR significance (B-H pass test)
Advanced liver inflammation (score ≥ 3)			
CCL3	0.45	0.041336	Yes
CCL4	0.47	0.035439	Yes
IL8	0.45	0.042123	Yes
CCR5	0.48	0.031463	Yes
IL8RB	0.50	0.024563	Yes
CCL21	0.50	0.024304	Yes
IFNA2	0.51	0.024371	Yes
IL19	0.51	0.020672	Yes
IL1F8	0.53	0.013993	Yes
Advanced steatosis (score ≥ 3)			
IL1F10	0.45	0.043652	Yes
IL8RB	0.49	0.025506	Yes
NASH			
CXCL12	−0.44	0.049062	Yes
CCL1	0.45	0.045748	Yes
CCR3	0.46	0.039522	Yes
CCR9	0.46	0.039522	Yes
IL5	0.44	0.049062	Yes
IL8RA	0.47	0.032636	Yes
IL8RB	0.44	0.049062	Yes
IL1RN	0.53	0.014784	Yes
IL9	0.53	0.014605	Yes
Fibrosis			
C5	0.46	0.038905	Yes
SCYE1	0.48	0.030164	Yes
IL1RN	0.53	0.015503	Yes

Advanced liver inflammation (score ≥ 3) ($N = 10$), advanced steatosis (score ≥ 3) ($N = 8$), NASH ($N = 13$), fibrosis ($N = 15$). NASH: nonalcoholic steatohepatitis.

NASH as compared to those NAFLD patients without NASH showed a significant differential expression of chemokine (C-C motif) receptor 3 (*CCR3*), chemokine (C-C motif) receptor 9 (*CCR9*), interleukin 1 receptor antagonist (*IL1RN*), interleukin 8 receptor α (*IL8RA),* and interleukin 9 (*IL9*) ($P \leq 0.05$) (Table 2). Spearman's correlation coefficient analysis showed some of the differentially expressed genes, namely, *CCR3, CCR9, IL1RN, IL8RA,* and *IL9* to be also positively correlated with NASH ($P \leq 0.05$) (Table 3). Additionally, *IL8RB*, chemokine (C-X-C motif) ligand 14 (*CXCL12),* and chemokine (C-X-C motif) ligand 1 (*CCL1*) were also positively and significantly correlated with NASH ($P \leq 0.05$) (Table 3).

3.4. Gene Expression Differences between Patients with and without Hepatic Fibrosis. In patients with hepatic fibrosis, only chemokine (C-X-C motif) ligand 17 (*CCL17*) was significantly upregulated ($P \leq 0.05$) (Table 2). A different set of genes, small inducible cytokine subfamily E member 1 (SCYE1), *IL1RN,* and complement component 5 (*C5*), however, were positively correlated with severity of fibrosis ($P \leq 0.05$) (Table 3).

3.5. Independent Predictors of Advanced Inflammation, NASH, and Fibrosis. To predict advanced hepatic inflammation, a single equation multivariate regression model was generated. In this model, only four variables—*CCL21, CCR5*, ALT, and age acted as predictors of advanced inflammation, where *CCL21* ($P < 0.0007$) and *CCR5* ($P < 0.0064$) were the strongest predictors (Table 4). These four predictors explain 66% of the variance in the inflammation phenotype ($R^2 = 0.66$).

For understanding the effect of independent variables on pathogenesis of histologic NASH, the multivariate regression generated a statistically significant model ($P < 0.002$) with *CCR3, CXCL12, IL1RN, IL8RA, IL8RB,* and interleukin 5 (*IL5*). This model explained 75% of the variance in NASH phenotype ($R^2 = 0.75$).

The model of advanced hepatic fibrosis ($P < 0.006$) included only *IL1RN* ($P < 0.006$) as a sole component explaining 34% of the variance in fibrosis ($R^2 = 0.34$). Interestingly, none of the genes showing differential regulation ($P \leq 0.05$) or significantly correlated with the degree of steatosis were able to contribute significantly to the model for steatosis; hence, no models resulted from these analyses.

4. Discussion

Liver is a major organ involved in lipid metabolism. However, it has limited capacity to store lipids [27]. Therefore, excess lipid buildup can result in the development of NAFLD. One of the critical thrusts in the studies of the progression of NALFD has been the search for factors that may influence the progression of steatosis to NASH and cirrhosis. According to the multiple hit model of NAFLD, many hits may act in parallel or in tandem contributing to this pathogenesis. Of these, gut-derived and adipose tissue–derived factors potentially play an important role contributing to inflammatory conditions, including NAFLD. Inflammation, a central player in the pathogenesis of NASH, can enhance the probability of progression of fibrosis to NASH-related cirrhosis [28].

In the past decade, white adipose tissue has been considered as a major source for inflammatory cytokines and chemokines in obese patients [29–31]. In addition to the adipose tissue, it was suggested that other tissues, particularly, gastric and intestinal tissues may overproduce various soluble molecules and contribute to overall inflammatory background influencing distant organs [31].

Our study is the first to show that mRNAs encoding for various soluble molecules are overproduced in the gastric tissue of morbidly obese patients with advanced forms of NAFLD. Remarkably, there was a substantial overlap in genes with significant differential expression ($P \leq 0.05$) and genes with significant correlation ($P \leq 0.05$) to the

Table 4: Best fitting multiple linear regression models showing the relationship between predictor variables and the predicted clinical parameter.

Group	Independent variable	Regression coefficient β	P values of independent variables	P value of the entire model
Advanced liver inflammation (score ≥ 3)	(Intercept)	−0.5745 ± 0.8110	0.4896	$P < 0.001$
	CCL21	0.0012 ± 0.0003	0.0007	
	CCR5	0.0004 ± 0.0001	0.0064	
	AGE	0.0288 ± 0.0162	0.0964	
	ALT	0.0267 ± 0.0136	0.0688	
NASH	(Intercept)	0.3545 ± 0.1988	0.0980	$P < 0.002$
	CCR3	0.0003 ± 0.0002	0.0626	
	CXCL12	−0.0001 ± 0.0000	0.0724	
	IL1RN	0.0003 ± 0.0001	0.0810	
	IL5	−0.0003 ± 0.0001	0.0683	
	IL8RA	0.0655 ± 0.0215	0.0092	
	IL8RB	0.0004 ± 0.0002	0.0532	
Fibrosis	(Intercept)	0.5750 ± 0.2471	0.0318	$P < 0.006$
	IL1RN	0.0007 ± 0.0002	0.0063	

Regression coefficient β represents slope estimate ± standard error of the estimate (SE) ($P \leq 0.05$ were considered significant).

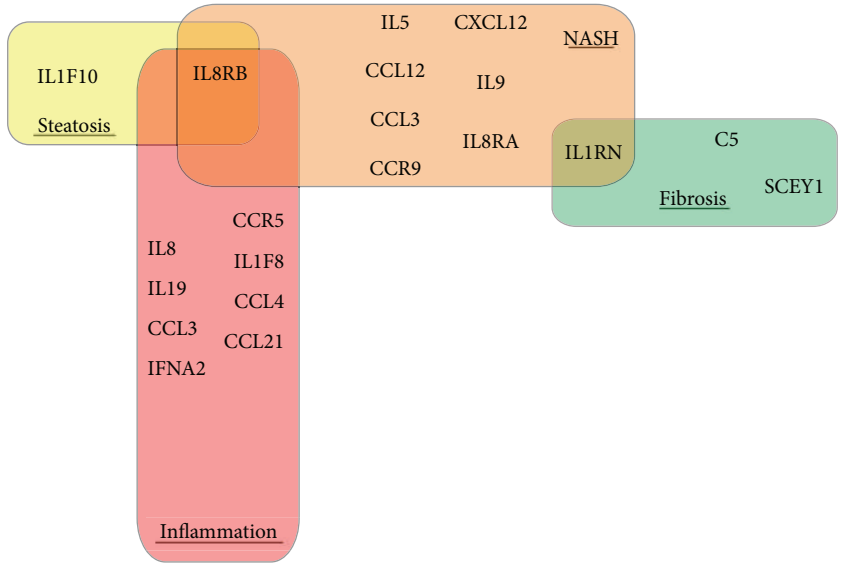

Figure 1: Venn diagram depicting results of an analysis of correlations. Sets of genes significantly correlating ($P \leq 0.05$) with specific histological characteristic of nonalcoholic fatty liver disease (NAFLD) overlap only minimally.

same histological characteristic of NAFLD (Supplementary Figure 1). Further, distinct and notably, nonoverlapping sets of soluble molecule encoding genes change their expression along with various histological features of NAFLD (Figure 1). Importantly, an overlap between sets of genes significantly correlating ($P \leq 0.05$) with a specific histological characteristic of NAFLD was minimal (Figure 1). *IL8RB/CXCR2* is a notable exclusion with its overexpression correlating with steatosis and diagnosis of NASH as well as fibrosis.

IL8RB/CXCR2 is a receptor for the IL8 chemokine that plays an important role in liver inflammation, regeneration, and repair [32, 33] as well as in the neutrophil accumulation in other inflammatory conditions [31, 34]. Increased levels of the gastric expression of *IL8RB* gene indicate that in morbidly obese patients with NASH-associated inflammation, IL8 activation is not limited to hepatic macrophages as had been shown before [32], but is a system-wide feature. It is plausible that IL8RB present on the resident gastric macrophages cells or on neutrophils activates the neutrophils locally upon its binding to IL8. In turn, activated neutrophils may then release additional chemokines and/or may enter the liver through portal circulation and influence the progression of NAFLD

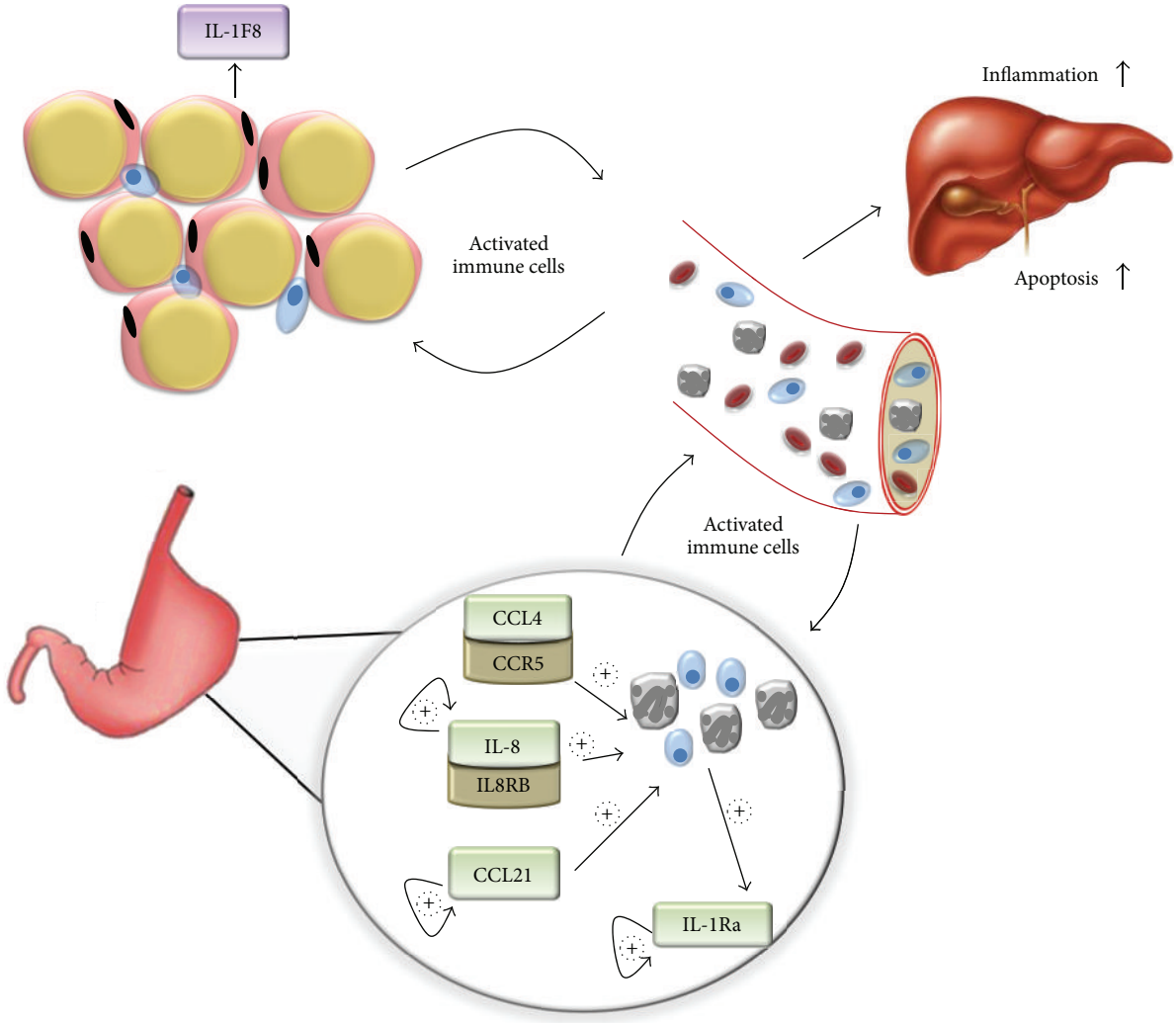

FIGURE 2: Inflammation-related genes in stomach and obesity-associated nonalcoholic fatty liver disease (NAFLD). In obesity, increased levels of inflammatory molecules such as IL1F8 may alter gene expression in stomach by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). NF-κB is known to activate gene expression of a number of downstream inflammatory molecules including CCL4, IL8, and CCL21. These inflammatory molecules may then regulate their own expression in positive feedback loop, thus further exacerbating inflammatory profile. These molecules can potentially activate local immune cells and attract additional immune cells. Oxidative stress triggered by activated immune cells can further add to existing inflammation. The entry of secreted inflammatory molecules and activated immune cells into portal circulation may contribute to NAFLD.

(Figure 2). This premise is also supported by our observation that the expression of *IL8* gene that encodes the ligand for IL8RB positively correlates with advanced hepatic inflammation (Table 3). Circulatory IL8 levels are reported to increase under oxidative stress and, in turn, stimulate further increase in levels of oxidant stress mediators by local recruitment of inflammatory cells [35] (Figure 2). As an expanding adipose tissue of obese individuals releases increased levels of IL8 [9, 30], it may trigger increased expression of gastric IL8 and its receptor IL8RB. Additionally, studies have shown that free fatty acids (FFA), also increased in obese individuals, influence expression of IL8 in various peripheral tissues [36, 37]. Thus, the paired increase in levels of IL8 and its receptor found in the gastric tissue of obese may act to activate local as well as circulating, thus contributing towards vicious cycle of inflammation and influencing progression of NAFLD.

The expression levels of anti-inflammatory receptor IL1RN, an antagonist of IL1A and IL1B, were positively correlated both with the presence of NASH and with fibrosis (Table 3). In the regression model predicting fibrosis, expression of *IL1RN* mRNA was the only significant component that explained 34% of the variance in fibrosis. Additionally, *IL1RN* mRNA expression significantly contributed to the regression model predicting NASH (Table 4). These observations are in agreement with a recent report on association of serum IL1Ra levels and liver *IL1RN* expression with NASH [38]. IL1Ra is expressed and secreted by a number of immune cells such as monocytes, macrophages, and neutrophils as well

Table 5: Validation of PCR array data by individual qPCR assays for the selected set of genes.

Genes	Fold change	P value
Advanced liver inflammation (score ≥ 3)/mild/no liver inflammation (score < 3)		
CCL4	1.5	0.01
CCR5	1.2	0.01
IFNA2	2.5	0.001
IL19	1.9	0.05
IL1F8	11.0	0.025
Advanced steatosis (score ≥ 3)/mild/no steatosis (score < 3)		
IL8RB	1.9	0.03
NASH/no NASH‡		
IL1RN	1.19	0.03
IL9	2.0	0.02

as epithelial cells and hepatocytes [39]. As its expression is regulated by proinflammatory cytokines, IL1RN is considered to be an acute phase protein [40] with levels elevated in many inflammatory conditions [41]. We hypothesize that increased levels of circulating and/or local proinflammatory cytokines upregulate gastric IL1RN expression either directly or via activated leukocytes (Figure 2). Once upregulated, IL1Ra may stimulate its own gastric expression by a positive feedback loop (Figure 2). This mechanism is supported by studies showing elevated circulating IL1RN in patients with obesity [40] and NAFLD [38, 42].

Many genes differentially expressed in the gastric tissue of patients with advanced forms of NAFLD encode chemokines previously shown as important players in a variety of inflammatory conditions. For example, expression levels of both *CCL4* chemokine and its receptor *CCR5* encoding genes showed significant upregulation in advanced hepatic inflammation (Table 2) and a positive correlation with the severity of the hepatic inflammation ($P \leq 0.05$) (Table 3). In the multivariate regression model, *CCR5* mRNA level also was one of the strongest predictors of the severity of hepatic inflammation (Table 4). CCL4 attracts natural killer cells, monocytes, and a variety of other immune cells [1]. The increased expression of *CCL4* and *CCR5* genes in gastric tissue could be attributed to local immune cells activated in response to upstream regulators like IL1F8 (Figure 2). In the present study, *IL1F8* gene was also upregulated in stomach tissue of patients with advanced liver inflammation (Tables 2 and 5). CCR5 has been implicated in NASH [43] and hepatic fibrosis [44]. Both of these conditions develop almost exclusively in a proinflammatory environment. While the role of CCL4/CCR5 in the pathogenesis of NAFLD remains to be sketched out, these collective findings make it an attractive target for further investigation.

The complex interaction of cytokines, chemokines, and their receptors highlighted in this study suggests that the gastric tissue is an integral player in obesity-associated NAFLD. It seems that in obesity, an increase in inflammatory responses of adipose tissue corresponds to similar increase in the inflammation within the tissues involved in satiety response. Activated immune cells embedded in the gastric tissue may then recruit additional immune cells or be released in circulation, and hence amplify the inflammatory response and promote the development and progression of NAFLD (Figure 2). An increase in recognition of the endocrine function of the stomach and its contributions to energy homeostasis prompts us to hypothesize that its altered inflammatory profile may influence its endocrine secretion. This, in turn, may trigger a cascade of metabolic dysfunction culminating in NAFLD (Figure 2). It remains to be determined if the complex interaction of inflammatory molecules in gastric tissue lies upstream or downstream of the intricate network of inflammatory signaling, which is the hallmark of NAFLD. Evidently, the stomach plays a certain role in metabolic dysfunction; its potential proinflammatory properties should not be neglected by studies of the conditions related to metabolic syndromes, including NAFLD.

5. Conclusion

In this study, we demonstrate an altered pattern of gene expression for cytokine and chemokine encoding genes in the gastric tissue of individuals with obesity and varying degrees of hepatic inflammation and different forms of NAFLD. Soluble inflammatory molecules produced by the stomach appear to contribute to obesity-related NAFLD. Although the causal links between these signaling events remains to be determined, we propose that the fundus of the stomach is an integral player in the signaling milieu associated with both obesity-related NAFLD.

Acknowledgments

This study was performed at the Translational Research Institute, as part of the collaborative effort between the George Mason University and the Inova Hospital. The authors would like to thank both their teams for their invaluable help and assistance.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 654963, 8 pages
http://dx.doi.org/10.1155/2013/654963

Review Article

Inflammasome in Intestinal Inflammation and Cancer

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Received 22 January 2013; Accepted 7 March 2013

Academic Editor: Amado Salvador Peña

The activation of specific cytosolic pathogen recognition receptors, the nucleotide-binding-oligomerization-domain- (NOD-) like receptors (NLRs), leads to the assembly of the inflammasome, a multimeric complex platform that activates caspase-1. The caspase-1 pathway leads to the upregulation of important cytokines from the interleukin (IL)-1 family, IL-1β, and IL-18, with subsequent activation of the innate immune response. In this review, we discuss the molecular structure, the mechanisms behind the inflammasome activation, and its possible role in the pathogenesis of inflammatory bowel diseases and intestinal cancer. Here, we show that the available data points towards the importance of the inflammasome in the innate intestinal immune response, being the complex involved in the maintenance of intestinal homeostasis, correct intestinal barrier function and efficient elimination of invading pathogens.

1. Introduction

In the human gut, trillions of bacteria interact with the host's systemic immune system in a complex balance between immune activation and tolerance [1]. Pathogen recognition receptors (PRRs) have been shown to play an important role in the differentiation between commensal and pathogenic bacteria [2]. The detection of pathogen molecules collectively known as pathogen-associated molecular patterns (PAMPs) by PRRs activates the innate immune system, being involved in infection recognition and its consequent inflammatory response [2, 3]. The activation of PRRs can lead to enhanced production of proinflammatory cytokines with a wide range of systemic and local effects. Among them, interleukin (IL)-1β has been shown to be secreted in high amounts by colonic monocytes from patients with active inflammatory bowel disease (IBD), and intestinal levels are consistently correlated with disease activity suggesting an important role of this cytokine in intestinal inflammation [4]. In addition, IL-1 has also been implicated in the promotion of angiogenesis, tumor growth, and metastasis in experimental cancer models, being associated with more aggressive tumor biology [5, 6].

Generation of IL-1β requires the activity of caspase-1, but the mechanism involved in the activation of proinflammatory caspases remained to be established until 2002. In that year, the group of the late Jürg Tschopp reported the identification of the inflammasome, a multimeric molecular platform which triggers the activation of inflammatory caspases and processes pro-IL-1β [13]. Inflammasomes are cytosolic multiprotein complexes activated by specific PRRs which are involved in infection recognition and inflammation [13–17]. The structure of the inflammasome is assembled by intracellular nucleotide-binding-oligomerization-domain- (NOD-) like receptors (NLRs) to initiate innate immune responses against invading pathogens activating caspase-l [15]. The subsequent activation of caspase-l leads to enhanced expression of IL-1β and IL-18, recruitment and activation of immune cells, and triggering of pyroptosis, a caspase-1-dependent inflammatory form of cell death [14, 18–21].

The importance of the inflammasome and the cell death programming associated with microbial invasion is to restrict pathogen growth and to activate and recruit immune cells to mediate host defense. As the activation of the inflammasome and the caspase-1 pathway leads to enhanced production of

cytokines known to be upregulated in IBD and cancer, the role of this pathway in intestinal inflammation and colonic neoplasia has been the subject of intense research in recent years.

2. The Inflammasome

Inflammasomes are composed of multiprotein cytosolic complexes that gather to activate caspase-1 [13]. These multimeric platforms are found in a wide range of cell types including macrophages, dendritic cells, adipocytes, keratinocytes, and epithelial cells [22–28]. These complexes are activated either by NLR proteins NLRP1, NLRP3, NLRC4, NLRP6, and NAIP5 or by the DNA-sensing complex of AIM2, a member of the interferon-inducible HIN-200 protein family. Activation of these receptors by certain PAMPs leads to their oligomerization and subsequent interaction with the adaptor protein ASC and the CARD domain of caspase-1. ASC, as well, presents a CARD domain that works together with the CARD domain of procaspase-1 [15]. Inflammasome-activated caspase-1 is then used for activation of the proinflammatory cytokines IL-1β and IL-18, both belonging to the IL-1 family. These inflammatory cytokines enhance antimicrobial functions of phagosomes and promote protection against intracellular pathogens [16] (Figure 1).

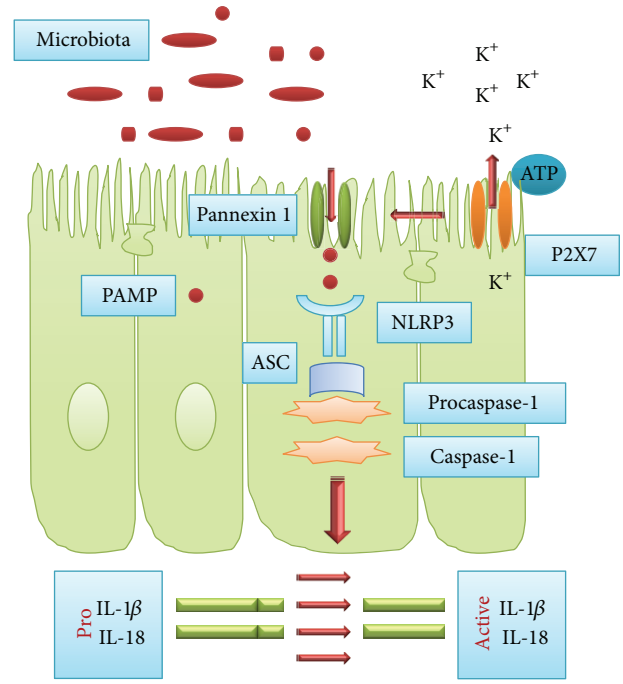

Figure 1: Inflammasome components, assembly, and activation. Microbial and nonmicrobial stimuli can induce the activation of the NLRP3 inflammasome. The NLRP3 inflammasome consists of a nucleotide-binding-oligomerization-domain- (NOD-) like receptor (NLR) that can be activated by certain bacterial toxins containing specific pathogen-associated molecular patterns (PAMPs), in the presence of extracellular adenosine triphosphate (ATP). Oligomerization of the NLR and ASC results in a macromolecular complex capable of cleaving procaspase-1 to its active form, which in turn cleaves the proforms of IL-1β and IL-18 to their biologically active forms.

3. Inflammasome and Inflammatory Bowel Disease

3.1. The Association between the Inflammasome and Inflammatory Bowel Diseases. Crohn's disease (CD) and ulcerative colitis (UC) are chronic immune-mediated inflammatory diseases of the gastrointestinal tract that result from a dysregulated mucosal immune response to bacterial antigens in the gut lumen of a genetically susceptible host [29, 30]. In the gut-microbiota interplay related to IBD pathogenesis, several previous findings point towards the potential role of the inflammasome in the development of chronic intestinal inflammation. The first evidence refers to the upregulation of inflammatory cytokines IL-1β and IL-18 in active IBD, and the discovery of IL-18 gene polymorphisms associated with CD [31–33]. The second is the presence of a dysregulated IL-1β production linked to CD and the association between the NLRP3 inflammasome and three rare autoinflammatory chronic disorders treated with Canakinumab, a human monoclonal antibody targeted at IL-1β [22, 31, 34, 35]. The third, and perhaps the most important evidence, is the association between the NLRP3 gene and CD in candidate-gene approach studies.

Genome-wide association studies (GWAS) have tried to dissect the inherited element of IBD, identifying more than 70 CD and 40 UC susceptibility loci [36, 37]. These studies, however, do not explain the majority of the heritability related to IBD [38]. One interesting genomic region not associated with IBD in GWAS, but pointed out in candidate-gene approach studies and gene expression analysis data, is the NLRP3 gene which encodes the NLRP3 or cryopyrin protein [39–41]. This protein is part of the NLRP3-inflammasome, and it has a pivotal role in the pathogenesis of other chronic inflammatory disorders as pseudogout, gout, and familial Mediterranean fever [42–44]. As a result of the genetic link between NLRP3 and CD, the NLRP3 inflammasome is the most studied caspase-1 inductor multimeric platform in the field of chronic intestinal inflammation.

In two independent candidate-gene studies, the NLRP3 gene was associated with CD [39, 40], but there was no association in a posterior large study from the UK [45]. Interestingly, the first study conducted by Villani and coworkers also performed functional assays to evaluate the impact of these polymorphisms in NLRP3 expression and IL-1β production [39]. In this regard, NLRP3 SNPs were associated with lower levels of NLRP3 m-RNA expression in a loss-of-function fashion with homozygosis for the risk allele being associated with the lowest level of NLRP3 expression in peripheral blood cells and monocytes. In addition, they observed an association between lower IL-1β levels and the risk NLRP3 allele in cultured monocytes in the presence or absence of lipopolysaccharide. In both cases, homozygosis for the risk allele was associated with the lowest level of IL-1β. Even though significantly higher IL-1β levels were found in the ulcerated intestinal mucosa from human CD samples

than in healthy controls, it was postulated that a dysregulated IL-1β production might play a role in CD pathogenesis for patients bearing these SNPs.

The second study identifying an association between polymorphisms in the NLRP3 and CD included 498 cases and 794 controls, reporting that variants of NLRP3 conferred susceptibility to CD in Swedish male individuals [40]. Even though an NLRP3 genetic susceptibility was found in this population, careful analysis of the results show that, differently from the study by Villani and coworkers, the NLRP3 SNP associated with CD was a gain-of-function polymorphism, possibly promoting the production of mature IL-1β with subsequent induction of caspase-1 activity. The authors postulate that patients with this specific NLRP3 polymorphism might present an increased susceptibility to CD as a result of an increased IL-1β production and not due to a dysregulation of the pathway. In addition, the risk for developing CD in this study was exclusively associated with male patients bearing variant alleles in both NLRP3 and CARD8 genes. A third study evaluating the association between NLRP3 polymorphisms and IBD added more controversy to the topic. Lewis and coworkers raised questions about the previously reported association between CD and the NLRP3 locus supported by negative results based on control allele frequency data from large GWA studies [45].

3.2. Inflammasome Activation in the Gut. Even though the role of the NLRP3 inflammasome in IBD is still a matter of debate, the mechanisms behind its function started to be recently unveiled. NLRP3 can be triggered by bacterial constituents, synthetic purine-like compounds, endogenous urate crystals, and exogenous adenosine triphosphate (ATP) [46–48]. Of note, it was postulated that the passage of bacterial molecules into the host cytosol leading to NLRP3 inflammasome activation can be mediated by pannexin-1 and $P2X_7$ receptor [49]. Pannexin-1 constitutes a transmembrane hemichannel that associates with $P2X_7$ receptor, a member of the ATP-activated P2X purinergic receptors family, permeable to monovalent cations and anions, and capable of inducing the opening of a larger pore permeable to hydrophilic macromolecules [50]. In particular, the $P2X_7$ receptors act as danger sensors in immune cells and have been implicated in different biological functions, including apoptosis and the production and release of proinflammatory cytokines [51].

In this regard, investigators have demonstrated that the cytosolic recognition of bacterial molecules resulting in the NLRP3 inflammasome activation is mediated by pannexin-1 activation [52]. These results seem to indicate that NLRP3 would function downstream of pannexin-1/$P2X_7$ receptor in response to bacterial components to regulate caspase-1 activation (Figure 1). Furthermore, the expression and site-specific modulation of $P2X_7$ receptors was demonstrated on epithelial and immune cells of the gut, supporting the suggestion of purinergic signaling as an additional component of the innate immune circuits involved in the control of inflammation and cell fate in the gut and gut-associated lymphoid tissues [53]. In addition, in intestinal epithelial cells, the expression of $P2X_7$ receptors was also found to be upregulated by interferon-gamma, a proinflammatory cytokine and a signature molecule of the Th-1 type of immune response [54]. Moreover, ATP was shown to induce apoptosis and autophagy in human epithelial cells, possibly via reactive oxygen species production, through activation of the $P2X_7$ receptor [55]. Taken together, these findings appear to implicate $P2X_7$ receptors associated with pannexin-1 and the consequent NLRP3 inflammasome activation in the pathogenesis of diseases based on the dysregulation of the immune response such as IBD.

3.3. Inflammasome and Intestinal Inflammation in Animal Models. As the main downstream impact of the activation of the inflammasome is the upregulation of IL-18 and IL-1β, the knockout of these two important inflammatory cytokines as well as of other upstream regulators is pivotal to fully understand the role of the inflammasome in intestinal inflammation. Therefore, genetically modified mice lacking IL-18, IL-18 receptor (IL-18R), IL-1 receptor (IL-1R), NLRP3, NLRP6, ASC, and caspase-1 were constructed. In general, the susceptibility of these animals to intestinal inflammation was tested using the dextran sulphate sodium (DSS) experimental colitis model.

In the context of DSS colitis models, the role of IL-18 and IL-1β is still a matter of debate. More recently, it has been shown that IL-18 and IL-18R knockout mice develop more severe inflammation compared to wild-type, which is not true for IL-1R knockout mice [56, 57]. In an infection mouse model with *C. rodentium*, however, IL-1R knockout was shown to present increase mortality with severe colitis characterized by intramural colonic bleeding and intestinal damage following infection [57]. Consistent with these findings, most recent studies present clear data that mice lacking NLRP3 are more susceptible to develop colitis [26, 58–60] and ASC and caspase-1-deficient mice present enhanced susceptibility to DSS-induced inflammation [61].

However, other studies have shown exactly the opposite—that transgenic or pharmacological blockage of IL-1β converting enzyme (ICE) or IL-18 ameliorate DSS colitis [62–65]. In keeping with these results, Bauer and coworkers reported decreased sensitivity to DSS in NLRP3 deficient mice [66]. In that study, IL-1β secretion was abrogated in macrophages lacking NLRP3, ASC, or caspase-1 confirming that DSS activates caspase-1 via the NLRP3 inflammasome. After administration of DSS, NLRP3 knockout mice developed less severe colitis than wild-type mice and produced lower levels of proinflammatory cytokines in colonic tissue. In addition, pharmacological inhibition of caspase-1 with pralnacasan achieved a level of mucosal protection equivalent to NLRP3 deficiency. More recently, this protective role of NLRP3 against DSS colitis was also demonstrated by yet another independent group [67]. In any case, regardless of the still debated role of the NLRP3 inflammasome in DSS colitis, it has been shown that NLRP3, ASC, and caspase-1 deficient mice do not develop colitis without DSS treatment, implying that isolated inflammasome impairment does not result in spontaneous intestinal inflammation [61].

Another inflammasome, NLRP6 [68], has been associated with IBD [11, 12, 67]. Consistent with the presumed role for NLRP6 in inflammasome signaling, Chen and coworkers have shown that mice lacking NLRP6 present decreased levels of serum IL-18 after DSS treatment [11]. These mice deficient in NLRP6 develop a colitis phenotype, and this is transmissible to cohoused wild-type mice, both early in postnatal life and during adulthood [67]. Upon injury, NLRP6 deficiency deregulates regeneration of the colonic mucosa and epithelial proliferation and migration. Consistently, an analysis on a whole-genome expression profiling revealed a link between NLRP6 and self-renewal of the epithelium [12]. The inability of mice lacking NLRP6 to repair damaged epithelium as efficiently as WT mice resulted in extended increase in epithelial proliferative activity [11].

Recently, the role of the inflammasome in gut-related infection and sepsis has also been addressed. For this purpose, it has been shown that mice treated with large-spectrum antibiotics before DSS intervention show symptoms of sepsis, not colitis, due to translocation of a pathogenic strain of *E. coli* [69]. This particular model is very significant due to its resemblance to the common clinical scenario in which patients undergoing antibiotic and gut-damaging cytotoxic treatments develop septicemia. In this antibiotics-DSS model, mice lacking NAIP5-NLRC4 presented highly attenuated disease progression when compared to controls. Similarly, caspase 1 and IL-1β deficient animals were protected from *E-coli* systemic inflammatory response showing that NAIP5-NLRC4 inflammasome signaling through IL-1β is important for the development of gut-related sepsis [69]. Locally, NLRC4-dependent IL-1β production by intestinal phagocytes represents a specific response discriminating pathogenic from commensal bacteria and contributes to host defense in the intestine [70]. Upon infection with pathogenic bacteria, intestinal phagocytes produce mature IL-1β through the NLRC4 inflammasome and mice deficient in NLRC4 or IL-1β receptor are highly susceptible to intestinal infection [70, 71]. It seems, however, that the inflammasome does not only signal through IL-1β or IL-18 in systemic inflammatory responses [72]. It has been shown that systemic inflammasome activation by flagellin leads to loss of vascular fluid into the intestine and peritoneal cavity and death in mice, and this outcome depends on NAIP5, NLRC4, and caspase-1 signaling, but is independent of IL-1β or IL-18 [72]. Instead, flagellin-related inflammasome activation results in a pathological release of signaling lipids, including prostaglandins and leukotrienes that rapidly initiate inflammation and vascular fluid loss.

4. Inflammasome and Colorectal Cancer

4.1. Colitis-Associated Tumorigenesis. The role of the inflammasome in cancer physiopathology is complex as it can either lead to inflammasome-dependent carcinogenic inflammation or play a role in the process of eliminating malignant precursors through programmed cell death [73]. Not only the product of the inflammasome activation, caspase-1, is associated with inflammation and carcinogenesis, but also it can stimulate immune responses against tumoral cells. In colonic tissue, the role of the inflammasome in colorectal cancer tumorigenesis was mainly explored using the azoxymethane (AOM) DSS model in which administration of DSS after initiation with a low dose of AOM exerts a powerful tumor-promoting inflammatory activity in colon in mice [74]. Using this inflammation-driven tumorigenesis model draws a parallel to the carcinogenic process that takes place in IBD-related intestinal neoplasia. In these models, it has been shown that the absence of inflammasome-related interleukins, mainly IL-18, can greatly impact carcinogenesis and tumor progression. IL-18-deficient mice, for instance, have increased inflammation and tumor development in a colitis-associated colon cancer model [8]. It seems, however, that IL-18 can also influence epithelial growth by regulating the production of additional interleukins. In this regard, activation of NLRP3 or NLRP6 inflammasomes leads to IL-18-dependent downregulation of IL-22 blocking protein (IL-22bp) and higher expression of IL-22. This IL-22-IL-22bp axis was shown to critically regulate intestinal tissue repair and tumorigenesis in the colon [75]. The main studies evaluating the role of the inflammasome in colitis-associated cancer using the AOM/DSS model are summarized in Table 1.

Mice lacking NLRP3 were shown to be more susceptible to tumorigenesis in the AOM-DSS model in some studies [7, 9], but not in others [10]. In studies that demonstrated a positive association, NLRP3 deficient mice presented more inflammation and higher tumor burden compared to controls. In these NLRP3 knockouts, colonic IL-18 levels were shown to be lower than those of controls. It was postulated, therefore, that IL-18 might be associated with colon protection against tumorigenesis. In this regard, knockout mice for IL-18 treated with AOM/DSS contained significantly more tumors than controls [7, 8]. Importantly, recombinant IL-18 was successfully used as rescue, being able to reverse disease progression perhaps through induction of IFN-γ and its antitumor signaling involving activation of the transcription factor STAT1 [7]. Of note, IL-18 uses MyD88 as a downstream signal transduction effector and MyD88 signaling has been shown to have a protective role in the development of AOM/DSS colitis [8]. It has been proposed that the increased susceptibility of IL-18 deficient mice to colitis and cancer in the AOM/DSS model may be partially dependent on MyD88-related mechanisms, although Il-18 deficient mice present a milder phenotype compared with Myd88 knockout mice (less tumorigenesis) implying that other MyD88-related pathways might act with IL-18 to minimize carcinogenesis [8].

In the negative study, there were no differences in tumor formation between NLRP3 deficient mice and controls after challenge with AOM-DSS [10]. In contrast, another inflammasome, NLRC4, was found to be associated with tumorigenesis in this model. In this regard, NLRC4 knockout mice had significantly increased tumor numbers and tumor load compared to wild-type animals, though no differences in inflammation severity were noted. Since NLRC4 is associated with p53-dependent apoptosis, it may provide a link to the increased tumorigenesis observed in caspase-1 deficient mice noted by three independent groups [7, 9, 10]. Caspase-1 has been shown to be associated with the regulation of

Table 1: Studies evaluating the role of the inflammasome in colitis-associated cancer using the AOM/DSS model.

Mice model	Background	Impact on cancer	Description	Publication
IL-18−/−	C57BL/6	Yes	Enhanced tumorigenesis	Zaki et al. [7] Salcedo et al. [8]
IL-18R−/−	C57BL/6	Yes	Enhanced tumorigenesis	Salcedo et al. [8]
IL-1R−/−	C57BL/6	No	No enhanced tumorigenesis	Salcedo et al. [8]
MyD88−/−	C57BL/6	Yes	Enhanced tumorigenesis	Salcedo et al. [8]
Caspase-1−/−	C57BL/6	Yes	Enhanced tumorigenesis	Zaki et al. [7] Allen et al. [9] Hu et al. [10]
ASC−/−	C57BL/6	Yes	Enhanced tumorigenesis	Zaki et al. [7]
Pycard−/−	C57BL/6	Yes	Enhanced tumorigenesis	Allen et al. [9]
NRRP-3−/−	C57BL/6	Yes	Enhanced tumorigenesis	Zaki et al. [7] Allen et al. [9]
NRRP-3−/−	C57BL/6	No	No enhanced tumorigenesis	Hu et al. [10]
NLRC4−/−	C57BL/6	Yes	Enhanced tumorigenesis	Hu et al. [10]
NLRP-6−/−	C57BL/6	Yes	Enhanced tumorigenesis	Chen et al. [11] Normand et al. [12]

colonic epithelial cell proliferation and apoptosis and not only inflammation per se. As a result, caspase-1 deficient mice show increased colonic epithelial cell proliferation in early stages of tumor formation and reduced apoptosis in advanced tumors [10]. Hu and colleagues studied caspase-1 mRNA expression levels in normal colon tissue and colon tumors from WT mice observing a significant reduction in caspase-1 mRNA expression levels in tumors compared to normal colonic tissue, suggesting that lack of caspase-1 may play a role in tumor progression [10]. Similarly to caspase-1 deficient mice, NLRC4 knockout mice features significantly enhanced proliferation in both steady state and the early phase of inflammation-induced tumor formation [10].

Another inflammasome, NLRP6, was also found to play a role in AOM-DSS tumorigenesis [11, 12]. In this regard, NLRP6-deficient mice developed significantly more tumors compared to wildtype mice after chemical induction. The increase in tumors in these mice correlated with higher levels of intestinal epithelial proliferation, hyperplasia, and an increase in proinflammatory cytokines such as TNFα, IL-6, and IL-1β. Protection against tumorigenesis by NLRP6 is conferred specifically by hematopoietic cells rather than intestinal epithelial or stromal cells as irradiated wildtype mice that were transplanted with NLRP6 deficient bone marrow had similar numbers of tumors as NLRP6 deficient mice. Additionally, NLRP6 deficient recipients that received wildtype bone marrow were significantly protected against tumorigenesis to a similar extent as wildtype animals [11]. These findings suggest that deficiency in NLRP6 function in hematopoietic-derived cells is important for NLRP6-mediated protection against colitis-induced tumorigenesis.

As sporadic and familial colorectal cancer tumorigenesis in humans is often caused by Wnt-activating mutations, Normand and colleagues performed a transcriptional profiling of tumoral and nontumoral biopsies from NLRP6 deficient mice and controls treated with the DSS-AOM regimen [12]. Within the set of 1,884 genes that were differentially expressed in NLRP6 deficient mice, a significant overrepresentation of paracrine actors of the p53 Wnt and Notch signaling pathways was observed, supporting the role of NLRP6 in regulation of intestinal crypt cell proliferation. Notably, the microarray analysis clearly revealed an overexpression of Wnt-signaling pathway genes in tumor resection specimens of NLRP6 deficient mice, particularly the proto-oncogene Mycl1.

4.2. The Inflammasome in the ApcMin Model. In mice derived from animals treated with ethylnitrosourea, a mutation was identified that predisposed to the development of spontaneous intestinal cancer [76]. This mutation was later found to be located in the APC gene, the mouse homologue of the human APC gene responsible for human familial adenomatous polyposis [77]. The development of the APC deficient mice was one of the first spontaneous genetic animal models for bowel cancer [78]. In this model, affected mice develop multiple adenomas throughout the entire intestinal tract at an early age. It has been shown that innate immune signaling has an important role in the intestinal tumorigenesis in this model. In this regard, Rakoff-Nahoum and Medzhitov have shown that MyD88-dependent signaling controls the expression of several modifier genes of intestinal tumorigenesis in ApcMin mice [79]. ApcMin mice that are also deficient in MyD88 have decreased number of polyps which are smaller in size than those in age-matched ApcMin mice. In the inflammasome field, there were attempts to evaluate the potential impact of caspase-1 signaling in the development of tumors in ApcMin mice with disappoints results as the crossbreeding between ApcMin and caspase-1 deficient mice does not impact the phenotype [80].

5. Conclusion

In summary, the activation of specific NLR inflammasomes was shown to be triggered by microbial molecules, whereas

defects in NLRs determine innate immune system abnormalities and changes in the intestinal microbiota. In particular, intestinal dysbiosis has been consistently linked to intestinal inflammation through defects of NLR family members. In conjunction, these data highlight the importance of the inflammasome in the innate intestinal immune response and the maintenance of intestinal homeostasis, with fundamental influence on barrier function and the efficient elimination of invading microorganisms. Therefore, the abnormal activation of the inflammasome, converging signals from the internal and external milieu, sensing diverse stressful and microbial elements, appears to position inflammasome as a critical mechanistic link in the context of chronic inflammatory disorders involving the gut.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 523170, 8 pages
http://dx.doi.org/10.1155/2013/523170

Review Article

Helicobacter pylori Infection, Chronic Inflammation, and Genomic Transformations in Gastric MALT Lymphoma

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Received 4 January 2013; Accepted 11 February 2013

Academic Editor: David Bernardo Ordiz

Nowadays, it is believed that the main role in the development of gastric mucosa-associated lymphoid tissue (MALT) lymphoma plays *Helicobacter pylori* infection. This world-wide distributed bacteria is in charge of most cases of not only upper gastrointestinal tract disorders but also some of extragastric problems. Constant stimulation of the immune system causes a B-lymphocytes proliferation, which is considered to be responsible for the neoplastic transformation. On the other hand, there are 10%–20% of patients who do not respond to *Helicobacter pylori* eradication treatment. This group has often a chromosome translocation, which suggests that there is another unknown, so far, pathogenetic mechanism of MALT lymphoma. Majority of genetic abnormalities are connected with nuclear factor-κB (NF-κB) pathway, which activates the uncontrolled proliferation of neoplastic cells. Translocations already described in studies are t(11;18)(q21;q21), which is the most common, t(14;18)(q32;q21), t(14;18)(q32;q21), and t(3;14)(p14.1;q32). This non-Hodgkin's lymphoma is an indolent type originated outside lymph nodes. In more than 50% of cases, it occurs in the stomach. Occasionally, it can be found in salivary and thyroid gland, lung, breast, bladder, skin, or any other place in the human body. This paper is a review of the current knowledge on etiology, pathogenesis, treatment, and follow-up of gastric MALT lymphoma.

1. Introduction

The name of mucosa-associated lymphoid tissue (MALT) lymphoma was first established in 1983 by Isaacson and Du [1]. From the beginning, it was adopted well and is still used in an unchanged form. Marginal zone lymphoma of MALT is, apart from diffuse large B-cell lymphoma, the most frequent type of lymphoma that occurs in the stomach. What is important is that it can develop in almost every organ and tissue, for instance lungs, breast, thyroid gland, bladder, skin, or orbital adnexa. It is an indolent type, but clinical outcomes and response to treatment vary among patients. MALT lymphoma arises from the extranodal sites reach in B-lymphocytes, which appears in response to chronic antigenic stimulation caused by infection (*Helicobacter pylori*) or autoimmune process (Hashimoto disease). This disorder is the best example of how infectious pathogens and genetic abnormalities lead to malignant transformation. Gastric MALT lymphoma pathogenesis is a complex process including many gene alternations that result in cancer appearance. Better understanding of the background of the disease is crucial for discovering new prognostic factors, helpful in deciding when more aggressive treatment should be employed.

2. Epidemiology

The incidence of malignant lymphomas is at the rate of 3%-4% of all malignancy worldwide and has been increasing during the last 50 years. Lately, some stabilization in the number of diagnosis was observed, but only in developed countries. Malignant lymphomas are observed to be more frequent in North America, Australia, and Europe than in Asia and Africa. MALT lymphomas determine almost 7% of all non-Hodgkin's lymphoma, and at least 40% is primarily located in stomach. It is confirmed that gastric MALT lymphoma occurs in younger patients than the rest

of malignant lymphomas. The MALT lymphoma is mainly a disease of older adults, with a median age of 60 years. There is a gentle predominance of females [2]. In Asia, there is much higher proportions of MALT lymphomas, which can be caused by more frequent prevalence of *Helicobacter pylori* in this region of the world.

3. Pathogenesis

3.1. Infectious Background. Gastric MALT lymphoma pathogenesis is strictly connected with *Helicobacter pylori* infection. Although 90% of population worldwide have confirmed bacteria colonization, only 2% will develop malignant lymphoma. It was confirmed by Weber et al. [3] that almost 90% of patients with gastric MALT lymphoma are infected with *Helicobacter pylori*. This curved bacillus, previously called *Campylobacter pyloridis*, is a Gram negative pathogen found in the stomach. It was discovered by Marshall and Warren in 1980s [4]. From the beginning, *Helicobacter pylori* was classified as a higher class I carcinogen. Although over 80% of people are asymptomatic, chronic infection can lead to gastritis, gastric and duodenal ulcer, gastric adenocarcinoma, and MALT lymphoma [5, 6]. Nowadays, it is widely accepted that *Helicobacter pylori* gastritis is crucial in an evolution of MALT lymphoma localized in stomach. It was confirmed by several studies that chronic gastric inflammation causes constant antigenic stimulation, which leads to clonal expansion of B-cell lymphocytes [7, 8].

In the gastric mucosal cells, there are elevated levels of some cytokines, including proliferation-inducing ligand (APRIL), which belongs to the tumour necrosis factor (TNF) family. The protein has a crucial role in B-cell maturation and survival. APRIL is produced by macrophages present in the gastric MALT infiltrate, located close to the neoplastic cells [9]. APRIL may also induce B-cells transformation and the progression to the diffuse large B-cell lymphoma (Figure 1). The survival and transformation of B cells in malignant lymphoma require additional signals. They come either from T cells or directly by the antigenic autostimulation of lymphoma cells [1]. Gastric inflammation causes the appearance of a large number of macrophages, which, under a *Helicobacter pylori* infection, release large amounts of APRIL. This mechanism may be enhanced and maintained by the activated T lymphocytes. Importantly, a number of APRIL-producing macrophages significantly decrease in complete remission after eradication therapy [9]. Thus, a new APRIL production-targeted therapy can be developed.

Other pathogens, are also suspected to play an important role in MALT lymphoma pathogenesis. There are bacteria such as *Campylobacter jejuni*, *Borrelia burgdorferi*, and *Chlamydia psittaci* and viruses like Hepatitis C virus (HCV) that are potentially responsible for oncogenesis. These pathogens were found in histological material, but so far no strong evidences were established [10].

3.2. Autoimmune Disease. Patients with autoimmune disease have for sure higher risk of developing MALT lymphoma. Autoreactive B cells infiltrate the healthy organs and create lymphoid infiltrate similar to normal MALT tissue with huge amount of reactive clonal B lymphocytes. This situation is observed in salivary gland in patients with diagnosis of Sjögren syndrome and in the thyroid gland in Hashimoto disease. Sjögren syndrome is associated with 44 times increased risk of lymphoma [11], whereas Hashimoto's thyroiditis causes 70 times increased risk of thyroid lymphoma [12].

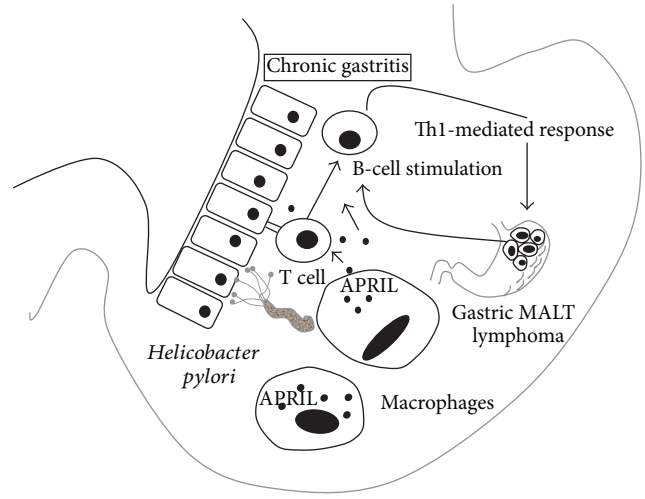

Figure 1: *Helicobacter pylori*-caused gastritis is crucial in an evolution of gastric MALT lymphoma localized in the stomach. In the gastric mucosal cells, there are elevated levels of some cytokines, including proliferation-inducing ligand (APRIL), the protein with a crucial role in B-cell maturation and survival. APRIL induces also B-cells transformation and the lymphoma progression. Gastritis attracted macrophages, which, under a *Helicobacter pylori* infection, release large amounts of APRIL.

3.3. Genetic Abnormalities. Gastric MALT lymphoma is connected with many genetic abnormalities and transformations. Some of them are proven to be strongly associated with the disease, but some are still not confirmed. It is believed, that on the background of chronic inflammation not only reactive B-cells are stimulated but also activated neutrophils which can lead to production of oxygen species. As a result, this genotoxins provoke DNA damages, which are responsible for mutations and transformations of genetic material.

The best known abnormality is t(11;18)(q21;q21), which was first described in 1989 [13]. It originates from a fusion of two proteins: apoptosis inhibitor 2 (API2) and paracaspase MALT lymphoma-translocation gene 1 (MALT1). It is extremely important that this translocation is present only in MALT lymphomas. What is more important is that while t(11;18)(q21;q21) is detected, no other chromosome abnormality can be found [14]. Unfortunately, positive cases do not response to *Helicobacter pylori* eradication, but, in contrast, they do not transform to more aggressive diffusive large B-cell lymphoma [15]. It is known that complete remission can be seen in at least 20% of patients with t(11;18)(q21;q21). The incidence of positivity for this translocation MALT lymphoma is at approximately 20% in Europe [16, 17] but

is not as common in the United States where only 5% are positive [18].

Another translocation is detected in only 5% of gastric MALT lymphomas. Patients with t(1;14)(p22;q32) or its variant t(1;2)(p22;p12) often have other genomic mutations. Moreover, it is usually connected with an advanced stage of disease and poor outcomes. *BCL-10* gene is relocated from chromosome 1 to 14, which in consequence triggers overexpression of Bcl-10 protein also known as CIPER, CARMEN, or mE10. In healthy organisms, higher expression is observed in lymph nodes, spleen, and testis. So far, it is believed that Bcl-10 protein expression is responsible for proliferative effects [19, 20].

The t(14;18)(q32;q21)(*IGH-BCL-2*) is commonly present in follicular lymphoma, in about 20% of diffuse large B-cell lymphoma and sometimes in chronic lymphocytic leukemia. Although this aberration is extremely rare in other types of lymphomas, it can be found in some cases of gastric MALT lymphoma. It was discovered that this aberration occurs more often in HCV-infected patients [21]. Bcl-2 is an antiapoptotic protein, which helps in survival and expansion of clonal B cells. So far, the role of t(14;18) in gastric MALT lymphoma is not fully understood. Overexpression of Bcl-2 is found not only in translocation positive patients but also in the negative ones. It is believed that similar to other types of lymphomas, *t(14;18)(IGH-BCL-2)* must coexist with other genetic abnormalities in order to develop neoplasm.

t(3;14)(p14;q32)(*IGH-FOXP1*) is a newly described abnormality present in patients with MALT lymphoma. This aberration causes overexpression of forkhead box (FOX)P1 mRNA and protein [22]. Accurate mechanism of how transcription factor FOXP1 leads to lymphogenesis is not fully discovered. The first study showed that positivity for this translocation is approximately 10% of all MALT lymphoma patients [22]. This abnormality is commonly found with other genetic aberrations. The most recent studies described the presence of t(3;14)(p14;q32) in diffuse large B-cell lymphoma, outside the lymph nodes especially [23, 24]. Only one study, so far, confirmed the existence of this translocation in gastric MALT lymphoma [25] which involved bad clinical outcomes.

In pathogenesis of MALT lymphoma, the above described translocation promotes oncogenesis by similar well-known mechanism. The majority of them involve the same pathway, which leads to antigen receptor-mediated activation of NFκB. This is a crucial transcript factor which plays a key role in MALT lymphogenesis [26, 27]. It regulates processes connected with B-cell development, growth, and survival by production of cytokines and growth factors, for example, TNF-α family (BAFF). Latest studies have shown that B-cell activation in MALT lymphoma can be strictly connected with TNF family. It can be also responsible for activation of cell apoptosis [28, 29]. It is observed that in patients with higher BAFF levels in serum, the prognosis and survival are much worse [30].

Based on recent knowledge about genetic abnormalities in gastric MALT lymphoma, there is a model of multistep pathogenesis. On the background of chronic inflammation and antigenic stimulation occurs genetic instability. As a result, many possible translocation and unbalanced aberrations are observed.

4. Symptoms and Diagnosis

The symptoms presented by patients with gastric MALT lymphoma are extremely unspecific. This causes difficulties with making final diagnosis and finding disease at an early stage. The signs of the disease are usually connected with involved location. Gastric MALT lymphoma can be long-time asymptomatic or associated with dyspepsia, abdominal pain, vomiting, diarrhea, obstruction, and nausea. Sometimes bleeding from gastrointestinal tract or even perforation may occur while extensive lesions are present. As a result, symptoms of anemia like paleness, weakness, or easy fatigue can be observed. B symptoms (weight loss, unexplained fever, and night sweats) in gastric MALT lymphoma are very rare, but the most common of the above is weight loss. A prompt diagnosis is crucial, but, unfortunately, it is usually made by incidence. Patients with early stage of disease have usually low tumor growth and minimal possibility to spread. The clinical course is indolent and there is a good response to the treatment. In contrast, patients with advanced stage of disease can undergo transformation to more aggressive lymphoma and may become resistant to treatment.

Not only symptoms but also endoscopic picture can be inconclusive. Difficulties often arise to differentiate between chronic gastritis or ulcer from an early-stage lymphoma. In order to confirm the diagnosis, a histopathologic evaluation of the gastric biopsies is indispensable. Routine histology and immunohistochemistry are required to correctly distinguish the disease. There always must be made PCR or FISH analysis for t(11;18), which is important to separate groups that will not respond to standard treatment. Characteristic for gastric MALT lymphoma are lymphoepithelial lesions (LEL) with the presence of mainly two types of cells: neoplastic centrocyte-like or small lymphoid. Occasionally, there can be seen atypical plasmacytic tumor cells. There is no specific immunohistochemical profile typical for gastric MALT lymphoma diagnosis. In 50% of patients, there is coexpression of CD43/BCL2. Neoplastic cells are positive for CD-20 and negative for CD-10, CD-23, and cyclin D1.

Moreover, *Helicobacter pylori* infection must be investigated. If it is negative in histochemistry, rapid urea breath test or fecal antigen test have to be made. Another analysis to prove absence of *Helicobacter pylori* infection is serological test for CagA antibodies and *Helicobacter pylori*-IgG antibodies [31]. Sometimes, there is possibility to detect other *Helicobacter* species, for example, *heilmannii* or *felis* [32].

5. Staging and Risk Factors

Before taking any decision on how aggressive the treatment should be, it is extremely important to perform a complete staging of the disease. What is more important is that risk factors and individual parameters, which can affect later therapy, are crucial. Medical history must include information about the age, time of the first symptoms, the family

history, and medical condition. The most important factor that we rely on during choosing method of treatment is clinical stage of the patient. During physical examination, it is important to remember about Waldeyer's ring, which is mandatory in every gastric lymphoma patient. Staging in gastric MALT lymphoma is similar to that in other types of lymphomas. According to recent European Society for Medical Oncology (ESMO) recommendations [33], it should include morphology with basic biochemical studies. If the blood cell count is lower, it can be caused by infiltration of bone marrow. Biochemical tests can detect liver or kidney problems, which can be important before the beginning of a chemotherapy. It can also detect mineral abnormalities which should be corrected before treatment. Lactate dehydrogenase (LDH) and β2-mikroglobulin are prognostic factors and will be abnormally high in patients with fast-growing tumor. Coagulogram is another important test which shows us if the blood is clotting properly. Every newly diagnosed patient should be examined in case of certain viral infections that can affect treatment, such as hepatitis B and C or human immunodeficiency virus (HIV).

In every case, computed tomography (CT) scans of neck, chest, abdomen, and pelvis, which are crucial to evaluate enlarged lymph nodes, should be performed. Core needle biopsy of bone marrow is made to diagnose possible infiltration of neoplastic cells. It was confirmed that 15% of gastric MALT lymphoma patients have lymphoma cells in bone marrow. Positron emission tomography (PET) has still not confirmed clinical necessity, but it can be extremely helpful in controversial cases. Moreover, during staging procedures of gastric MALT lymphoma, gastroduodenal endoscopy must be made. Biopsies are taken from different sites of gastrointestinal tract (e.g., stomach, duodenum, and gastroesophageal junction) and every location that looks suspicious.

There is no special staging scale for gastric MALT lymphomas. Most often, Ann Arbour staging is employed, which describe the extend of all types of non-Hodgkin lymphoma in adults. This classification was modified by Musshoff et al. [34]. Thus, staging of gastric lymphoma based upon the Ann Arbor system includes stage I E, which is disease limited to the stomach without nodal spread. Stage II E_1 is tumor in the stomach with spread to adjacent contiguous lymph nodes. Stage II E_2 is tumor in the stomach with spread to lymph nodes that are noncontiguous with the primary tumor. Moreover, if the spleen is affected, we add S. If the person has any of the B symptoms, we add letter B, and if is asymptomatic, we assign A (Table 1).

Prognostic factors in gastric MALT lymphoma are similar to the value for non-Hodgkin B-cells lymphoma. Factors that determine poor outcome are age, high level of LDH in serum, higher ECOG performance status, stages III and IV in Ann-Arbour scale, white blood count, and more than one extranodal site. It was observed that patients with nodal invasion has difficulty with complete remission after eradication treatment.

Some genetic abnormalities are thought to be bad prognostic factors. For instance patients, with t(11;18)(q21;q21) especially, are resistant to the first line therapy, and remission rate was lower than that in patients of *API2-MALT1* negative (78% versus 22.2%; $P = 0.0001$) [35]. That is why followup is so important in this group. Only one study so far proved that the presence of t(3;14)(p14;q32) is connected with poor clinical outcomes of patients with gastric MALT lymphoma [25].

Table 1: Ann Arbour clinical staging for gastric lymphoma.

Stage	Localization
I E	Confined within the gastric wall
II E_1	Involvement of stomach and contiguous lymph nodes
II E_2	Involvement of stomach and noncontiguous subdiaphragmatic lymph nodes
III	Lymph nodes on both sides of the diaphragm
IV	Visceral metastasis or second extranodal site

Subscripts that can be added to staging:
E: extranodal, when lymphoid tissue outside lymph nodes is involved.
X: is added when the largest diameter is above 10 cm (called bulky disease).
S: is added when the spleen is involved.
A or B: B is added when one of B symptoms is present, and A is for asymptomatic patients.

6. Treatment

While *Helicobacter pylori* plays a main role in the pathogenesis of MALT lymphoma, it is also crucial in approach to the treatment. According to current international guidelines, first line treatment for localized *Helicobacter pylori*-positive patients should be dual eradication therapy [36–38]. The treatment may be used with every highly effective antibiotics against *Helicobacter pylori*, taking into consideration the locally expected antibiotic resistance. If there is no response to the therapy above, second line triple or quadruple therapy is used. It was reported that after two lines of treatment, 99.8% of patients were cured from gastritis [39]. In a large study of 1408 patients, remission after eradication treatment in early stage was observed in 77.5%.

Unfortunately, in 5%–10% of gastric MALT lymphoma patients, we cannot confirm *Helicobacter pylori* infection. Moreover, more than 30% patients are resistant to first line treatment, and 30% of them have t(11;18)(q21;q21). Treatment for this patients should be chosen individually depending on the clinical stage of disease. For those who have stable disease without any symptoms, the best approach is "watch and wait." This approach will be valid for older patients with comorbidities. Potential risk factors like molecular markers should be taken into consideration as well. Aggressive therapy should be considered in symptomatic or progressive disease. Radiotherapy, chemotherapy, and/or surgery can be considered after unsuccessful eradication treatment. Further recommended therapy in this group has not been established so far.

Surgery is considered to be a standard therapy in therapy of patients with gastric MALT lymphomas, but, recently, the value of this therapy has been not confirmed. Even if the lymphoma is localized at early stage, the gastrectomy should be rather extensive due to the nature of the disease. Sometimes

Table 2: Chemo/immunotherapy lymphoma as a second line treatment in gastric MALT, phase II trials.

Authors	Treatment	*n*	CR	PR	SD
Nakamura et al. 2005, [45]	Cyclophosphamide	12	83%		17%
Raderer et al. 2005, [49]	Oxaliplatin	4	56%	38%	6%
Jäger et al. 2006, [50]	Cladribine	19	100%		
Martinelli et al. 2005, [51]	Rituximab	27	46%	31%	
Conconi et al. 2011, [52]	Bortezomib	13	46%	15%	31%
Zinzani et al. 2004, [46]	Fludarabine and mitoxantrone	20	100%		
Raderer et al. 2006, [53]	R-CHOP (15 patients) R-CNOP (11 patients)	26	77%	23%	
Troch et al. 2013, [54]	Rituximab and cladribine	40	58%	23%	13%
Kiesewetter et al. 2012, [55]	Lenalidomide	18	33%	27.8%	16.7%

n: number of patients, CR: complete response, PR: partial response, SD: stable disease.
R-CHOP: rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone.
R-CNOP: rituximab plus cyclophosphamide, mitoxantrone, vincristine, and prednisone.

further treatment is still required. Moreover, it is a major surgery and can be associated with serious complications and worsen a quality of life. German Multicenter Study Group (GMSG) presented no difference between survival in patients treated with gastrectomy compared to eradication (overall survival rate 82% to 84%) [40]. What is more important is that there were observed 50% long-term complications were observed after surgery [41].

In few studies, there was confirmed an excellent disease control by using radiotherapy. The use of a modest dose of involved fields was performed on resistant-to-eradication therapy patients with early-stage disease. The dose was 25–35 Gy to the stomach and perigastric nodes for the period of 4 weeks [42, 43]. Compared to surgery, no serious long-term complications and toxicity were observed. Only nausea and anorexia were present during the time of radiotherapy.

For a long time it was believed that gastric MALT lymphoma is just a localized disease and that surgery and radiotherapy are the best treatment strategy. Now, when it is well known that it is disseminated disorder chemotherapy, it became more important. Still, there are no standard recommendations for relapse or progressive patients after therapy and for those with late stage of the disease from the beginning. It was observed that chemotherapy alone is more effective than surgery apart from some cases with gastric obstruction [44]. Many chemotherapeutics are tested. The most commonly used are alkylating agents, nucleoside analogs in combination with corticosteroids. Complete remission (CR) after oral monochemotherapy with cyclophosphamide was 83% in a study by Nakamura and coworkers [45]. Unfortunately, patients with positive translocation t(11; 18) are resistant to second line therapy with oral monochemotherapy with alkylating agents. Nucleoside analogs are confirmed to be effective in treatment of different kinds of indolent lymphomas. A polychemotherapy with fludarabine and mitoxantrone (FM) has a very good effect on patients with gastric MALT lymphoma in both first and second line treatment. All groups that consisted of 20 people achieved complete remission [46]. Also the role of cladribine or 2-chlorodeoxyadenosine (2-CdA) was investigated. The complete remission after 4 cycles achieved 84% of investigated and all of them reacted to the treatment [47]. It is important that patients with translocation t(11;18) respond to therapy as well [48]. After 2-CdA, there were observed complications such as toxicities of 3 and 4 grade of WHO, mainly leukopenia, infections, and secondary neoplastic disease. There are highly effective drugs which should be individually considered in each patient.

Nowadays, immunotherapy became an extremely important part of treatment of non-Hodgkin lymphomas. The most commonly used is rituximab. It is a chimeric mouse/human monoclonal antibody specified to CD20 antigen expressed on the surface of B lymphocytes. Firstly, its effectiveness was shown in follicular lymphoma [56]. Now it is widely used alone or in combination with chemotherapeutic drugs in many types of B-cell non-Hodgkin lymphomas. Rituximab binds to CD20 antigen and activates the lysis of B cells by mediating cytotoxicity of complement dependent (CDC) and cell-mediated cytotoxicity antibody dependent (ADCC). It is also believed to induce cell death by apoptotic mechanism. The role of this drug is still not clear in gastric MALT lymphoma. In 2003, there was a first-phase study by Conconi et al. [57] with rituximab in monotherapy in patients at any stage. The CR was observed in 29% and overall response rate (ORR) was 64%. The toxicity of this treatment was moderate or even mild, but the relapse rate was 36%. An important fact is that patients with translocation t(11;18) are responsive to rituximab treatment [58, 59]. What is more important is that in a study by the International Extranodal Lymphoma Study Group (IELSG), it was confirmed that chlorambucil in combination with rituximab was more effective than chlorambucil alone [60]. Also in phase II clinical trial by Troch et al. CR by rituximab with cladribine was achieved by 58% of patients [54]. The conclusion is that rituximab may a benefit in individual patients, but for the majority it is not sufficient when used alone. It is more effective in combination with standard chemotherapeutics.

The efficacy of the combination of rituximab with chlorambucil was evaluated in a randomized study (comparator was chlorambucil alone) by the International Extranodal Lymphoma Study Group (IELSG) in gastric MALT

lymphomas that had failed antibiotics and in nongastric MALT lymphomas. The preliminary report [40] showed that the 5-year event-free survival was significantly better for patients treated with chlorambucil plus rituximab. There were also studies by Raderer et al. with cycles generally used in more aggressive lymphomas. Twenty-six patients were administrated rituximab plus cyclophosphamide, doxorubicin or mitoxantrone, vincristine, and prednisone. Complete remission was observed in 77% and partial remission was achieved in 27% [53]. Lately, bortezomib, the first therapeutic proteasome inhibitor, was examined by Kiesewetter et al. in 2012 with CR in 33% and PR in 27.8% [55]. The results on phase II studies with chemotherapy and immunotherapy are shown in Table 2.

Still the place of autologous hematopoietic stem cell transplantation is unknown. So far, it is not a standard for treatment of indolent lymphomas. Outcomes in gastric MALT lymphoma patients with progressive, disseminated disease are very comparable with outcomes in follicular lymphoma.

7. Followup

Although gastric MALT lymphoma has a very favorable outcome, it is still important to have a proper followup. It is possible that the disease will return even after 5 years of complete remission. The relapse can be due to reinfection of *Helicobcater pylori*. In a study by Zullo et al., reinfection was observed in 2.7% [61]. The followup is obligatory in patients with gastric MALT lymphoma to identify early phase of the recurrence of the disease. To confirm a complete remission, there should be done both endoscopic and histological examination. Although, there are no specified recommendations for a followup, the biopsy of gastric sites should be made every 6 months in first two years, and later once a year for the next five years. Systemic followup consist of blood tests and minimal adequate radiological and ultrasound and should be made at least once a year in the first 5 years. The most common are chest X-ray and abdomen ultrasound. The transformation in more aggressive lymphoma is low at the level of 0.05% [35], but there is a higher risk of occurrence of secondary neoplasm [62] and gastric cancer [63]. These studies confirm that patients with gastric MALT lymphoma need a long-term followup not only to detect early recurrence but also to find secondary disease.

8. Conclusions

Recently, enormous progress has been made in better understanding of pathogenesis of gastric MALT lymphoma. Many important chromosome aberrations, such as t(11;18), have been detected. It has a great influence on the development of new and more effective treatment strategy. There still remain cellular and molecular routes that need to be explored and clarified. Still, not enough clinical trials are performed due to rare expression and high effectiveness of first line treatment of gastric MALT lymphoma. What is more important is that early diagnosis of gastric MALT lymphoma is extremely important. While the symptoms are unspecific or not, always during the endoscopic exam the complete histological biopsies must be taken to make diagnosis correctly. The less advanced the stage of the disease, the bigger the chances to achieve complete remission.

Acknowledgments

This work was supported under Grant no. 503/8-093-01/503-01 from the Medical University of Lodz, Poland.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 237921, 16 pages
http://dx.doi.org/10.1155/2013/237921

Review Article

The Role of Cell Surface Architecture of Lactobacilli in Host-Microbe Interactions in the Gastrointestinal Tract

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Received 30 December 2012; Accepted 11 February 2013

Academic Editor: David Bernardo Ordiz

Lactobacillus species can exert health promoting effects in the gastrointestinal tract (GIT) through many mechanisms, which include pathogen inhibition, maintenance of microbial balance, immunomodulation, and enhancement of the epithelial barrier function. Different species of the genus *Lactobacillus* can evoke different responses in the host, and not all strains of the same species can be considered beneficial. Strain variations may be related to diversity of the cell surface architecture of lactobacilli and the bacteria's ability to express certain surface components or secrete specific compounds in response to the host environment. Lactobacilli are known to modify their surface structures in response to stress factors such as bile and low pH, and these adaptations may help their survival in the face of harsh environmental conditions encountered in the GIT. In recent years, multiple cell surface-associated molecules have been implicated in the adherence of lactobacilli to the GIT lining, immunomodulation, and protective effects on intestinal epithelial barrier function. Identification of the relevant bacterial ligands and their host receptors is imperative for a better understanding of the mechanisms through which lactobacilli exert their beneficial effects on human health.

1. Introduction

The human gastrointestinal tract (GIT) is the body's largest interface with the environment and is a dynamic barrier that harbours a complex microbial community. The intestinal epithelium allows the uptake of nutrients, secretes water and electrolytes, and simultaneously acts as a barrier to exclude pathogens and toxins [1]. Humans and their symbiotic bacteria have co-evolved and their mutual interactions are essential for human health and well-being [2]. There is increasing experimental evidence for the role played by intestinal bacteria in modulating development of the host immune system and the barrier properties of the intestinal epithelium [3].

Lactobacilli are important in the food and fermentation industries. They are also frequently used as probiotics in foods, cultured milks, and various pharmaceutical preparations [4–6]. The presence of lactobacilli is important for maintenance of the intestinal microbial ecosystem and for providing protection against pathogen infection [7–9]. Lactobacilli are present throughout the GIT in varying proportions. They are dominant in the proximal small intestine [10], a nutrient rich environment, whereas in the faecal microbiota they are present at most ~0.01%–0.6% and this proportion varies significantly between individuals [11, 12]. They have the ability to adhere and interact with the epithelium and the mucosal layers, while surviving the hostile conditions of the luminal environment and the competing microbiota [13]. These properties add to their potential to be used as probiotics that fit the parameters set by the Operating Standards in 2002 (FAO/WHO: Guidelines for the evaluation of probiotics in food). However, studies have shown that different strains

of lactobacilli can evoke different responses in the host and therefore, the results from one strain cannot be generalised to others [9].

Adherence of lactobacilli to the intestinal epithelium is an important characteristic as it promotes persistence time and colonisation, stimulates microbe-host interactions through immunomodulation, and provides protection to the intestinal barrier by various mechanisms including antagonistic activities against pathogens [14]. Bacterial cell surface components (adhesins, polysaccharides, and proteins) play major roles in the adherence of lactobacilli to the intestinal epithelium, interactions that might lead to pathogen exclusion and immunomodulation of host cells [15, 16]. The adhesive properties of lactobacilli are directly linked to their surface properties which are influenced by the structure and composition of their cell wall. Several studies implicate cell surface components, either individually or collectively, in microbe-host interactions [17, 18].

Lactobacilli show great diversity in cell surface architecture and are known to modify their surface properties in response to environmental changes [19, 20]. Different macromolecules constituting the cell wall of lactobacilli have been shown to contribute to maintaining bacterial cell integrity during environmental stress [21]. The cell surface architecture of lactobacilli and their ability to express certain surface components, or to secrete specific compounds that act directly on the host cells, may thus influence the physicochemical properties of the bacterial cell and strain-specific properties.

This paper will focus on cell surface components of lactobacilli that influence host response and impart strain-specific characteristics to lactobacilli.

2. Cell Surface Structures

The cell envelope of lactobacilli, like that of all lactic acid bacteria, is composed of the bilipidic plasma membrane with embedded proteins encompassed by the cell wall. The bacterial cell wall consists of a thick multilayered sacculus made of peptidoglycan (PG), decorated with teichoic acids (wall teichoic acids (WTA) and/or lipoteichoic acids (LTA)), exopolysaccharides (EPS), proteinaceous filaments called pili, and proteins that are anchored to the cell wall through different mechanisms (Figure 1). Some species of lactobacilli display an additional paracrystalline layer of proteins surrounding the PG layer, referred to as the S-layer. These macromolecules together may play crucial roles in determining species and strain-specific characteristics of lactobacilli by influencing host-microbe interactions and microbial adaptations to the changing host environment.

2.1. Peptidoglycan. PG is the largest component of the bacterial cell wall and is an essential polymer in lactobacilli that determines the shape and preserves the integrity of the bacterial cell. The PG layer has been described as a fisherman's net, functioning both as a container for and a sieve to the bacteria [24]. The elastic nature of PG helps withstand stretching forces caused by bacterial turgor pressure, excludes

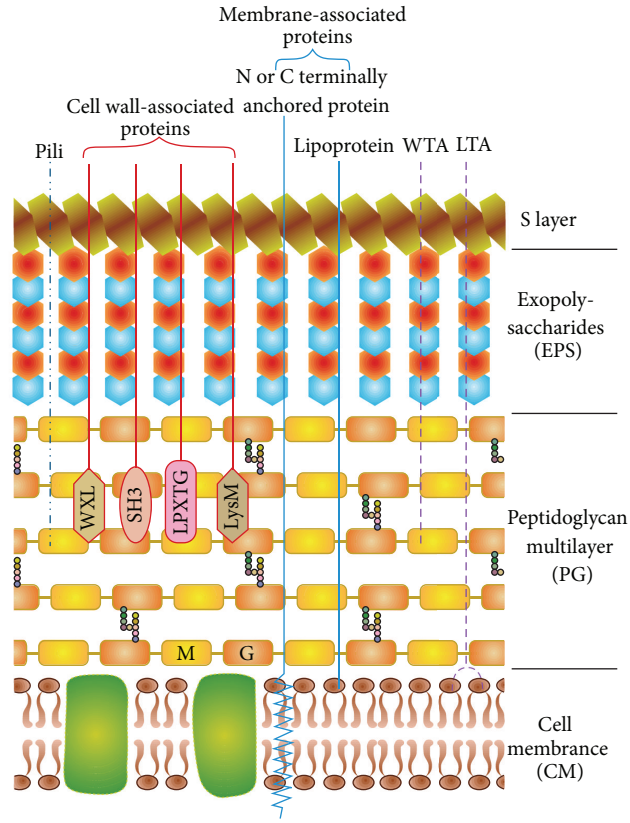

Figure 1: Cell envelope of lactobacilli with a schematic representation of cell-wall and membrane-associated proteins (the figure was adapted from [35, 153]). The bilipidic cell membrane (CM) with embedded proteins is covered by a multilayered peptidoglycan (PG) shell decorated with lipoteichoic acids (LTA), wall teichoic acids (WTA), pili, proteins, and lipoproteins. Exopolysaccharides (EPS) form a thick covering closely associated with PG and are surrounded by an outer envelope of S-layer proteins. The proteins are attached to the cell wall either covalently (LPXTG proteins) or noncovalently (exhibiting LysM, SH3, or WXL domains), lipid anchored to the CM (lipoproteins) or attached to the CM via N- or C-terminal transmembrane helix. M: N-acetyl-muramic acid; G: N-acetyl-glucosamine.

large molecules from entering the bacterial cell, and at the same time restricts secretion of large proteins. Proteins with theoretical molecular mass as large as 49.4 KDa and 82.1 KDa have been reported to be secreted by *Lactobacillus rhamnosus* GG and *Lactobacillus plantarum,* respectively [25, 26]. Some large proteins are unable to diffuse through the cell wall and are dependent on the cell wall expansion process to be dragged to the outer surface of the thick PG layer before being passively released into the external milieu [24, 27]. The threads of this net are polymers of covalently linked alternating residues of N-acetyl-glucosamine (GlcNAc) and β-1-4-linked N-acetyl-muramic acid (MurNAc). The glycan strands are held together by crosslinking pentapeptide side chains providing elasticity to the net. The pentapeptide side chain is made of alternating L- and D-amino acids and this attaches to the D-lactyl carboxyl group of MurNAc. Considerable variations occur in the basic compositions of

the glycan strands and pentapeptides which impart strain-specific characteristics to the bacteria [28, 29]. Following biosynthesis, assembly, and incorporation of the PG subunits, modifications in the GlcNAc and MurNAc structures can occur and affect interactions between host and lactobacilli [24]. These modifications include removal of acetyl groups from cell wall PG [30], 6-O-Acetylation of cell wall MurNAc residues [31], and the substitution of C6 of MurNAc by teichoic and teichuronic acids [32]. These modifications can affect the physiology of the bacterial cell wall by increased sensitivity to autolysis, resistance to lysozyme, and hydrophobicity of the cell envelope which in turn affects recognition by host receptors and bacterial adhesion [33, 34].

2.2. Teichoic Acids. Teichoic acids (TAs) are the second major component of cell walls of lactobacilli and account for up to half of the cell wall dry weight [35]. They are anionic polymers made of repeating units of glycerol- or ribitol-phosphate, covalently linked to PG as WTA or attached to the cytoplasmic membrane through their lipid anchors as LTA [36–38]. A fraction of LTA can be found free in the cell wall or may be released into the extracellular medium through deacetylation of the lipid anchor, where they are recognised as ligands by receptors present on intestinal epithelial cells [3]. LTAs contribute to the anionic character of the cell wall and provide hydrophobicity, which in turn influences the adhesiveness of the cell wall [34].

The overall structure of TA is a chain made of phosphodiester-bound glycerol or ribitol residues hooked through a terminal "linkage unit" on the C6 of the MurNAc residue of a growing PG chain. The structure of the linkage unit is well conserved and is made of a disaccharide N-acetylmannosaminyl β (1–4) glucosamine followed by glycerol phosphate. The variety of TA can occur in the nature of the sugars and number of phosphate residues. There are considerable variations in structure and abundance between WTA and LTA molecules. Their size and physicochemical properties depend on several factors such as species or strain, stage or rate of growth, availability of phosphate, acidity of medium, and carbon source, and so forth [24]. Although all lactobacilli have TA in their cell walls, not all *Lactobacillus* cell walls contain WTA and some species appear to contain only LTA [39]. TA can function as a reservoir for phosphates and also as a scavenger of cations (Mg^{++} in particular) [40, 41]. TAs can also help in creating a pH gradient across the cell wall and are also known to be involved in phage adsorption and autolysin activity [42]. Glycosylated TAs have been reported to be essential for the adsorption of some *L. plantarum* phages, and studies with *L. delbrueckii* subsp. *lactis* show the involvement of LTA in phage inactivation [43].

2.3. Cell Wall Polysaccharides. Cell wall polysaccharides are neutral polysaccharides that can either form a thick outer capsule closely associated with the cell wall and often be covalently bound to MurNAc of PG (referred to as capsular polysaccharide; CPS) or be loosely associated with it (wall polysaccharides; WPS) or be released into the extracellular medium (EPS) [44, 45]. Distinction between these various classes of cell wall polysaccharides is often difficult. In lactobacilli, EPS usually refers to extracellular polysaccharides that can be attached to the cell wall or released into the surrounding medium. The complex variations in the composition of EPS, which differs in the nature of the sugar monomers along with their linkages, distribution, and substitution, add to the structural variety of the *Lactobacillus* cell wall [46, 47]. EPS is generally composed of heteropolysaccharides consisting of different sugar moieties such as glucose, galactose, rhamnose, GlcNAc, and N-acetylgalactosamine [48]. Residues of glucuronic acid, phosphate, acetyl, and pyruvate groups may also be present in some strains of lactobacilli. In addition to the heteropolymeric EPS molecules, some strains of lactobacilli are capable of synthesising homopolysaccharides such as glucans or fructans from sucrose [49].

Studies with *L. rhamnosus* GG identified two different classes of EPS: long galactose rich molecules and short glucose/mannose rich EPS molecules [50]. Some polysaccharide chains can also be present as glycoproteins, providing anchorage to S-layer proteins, creating an extra level to the complexity of the bacterial cell wall architecture [50]. Specific contributions of EPS to cell wall functionality are unclear, although their general role is to mediate interactions of lactobacilli with environmental components and promote bacterial adhesion and biofilm formation to inert or living surfaces [51, 52].

2.4. Pili and Flagella. Pili are multisubunit protein polymeric structures that have been functionally analysed and characterised only in *L. rhamnosus* GG [53, 54], although they have been identified at the genome level in some lactobacilli [23]. These nonflagellar appendages are an assembly of multiple pilin subunits that are covalently coupled to each other by the transpeptidase activity of the pilin-specific sortase [53, 55]. The resulting isopeptide bonds are formed between the threonine of an LPXTG-like motif and the lysine of YPKN pilin motif in the pilin subunits [56]. After assembly, the pilins are attached to the cell wall by a membrane bound transpeptidase, the housekeeping sortase [57]. The roles of pili in bacterial adhesion, invasion, aggregation, formation of biofilms, and modulation of immunity are well established [58, 59] but the receptors in the host that recognise these pili are still unknown and their function in signalling host response is unclear. The presence of flagella is an unusual feature found in lactobacilli and at present, at least twelve motile species of lactobacilli have been recognised [11]. The bacterial flagellum comprises of polymers of protein called flagellin, which is suggested to act as a ligand and mediate activation of signalling pathways and modulation of host immune cells [60].

2.5. Cell Surface Proteins. The cell surface proteins in lactobacilli are either anchored to the cell wall by various mechanisms or secreted from the bacterial cell into the surrounding medium, where they reassociate with the cell wall through electrostatic interactions [61]. Cell surface proteins include the S-layer proteins which constitute the major cellular proteins that surround the cell. Examples of cell surface

proteins include the 43 KDa collagen binding S-layer protein from *L. crispatus* and cell surface proteins of 15 kDa and two proteins of 45 and 58 KDa from *L. acidophilus* CRL639 that are involved in binding to fibronectin and collagen [62]. Covalently anchored proteins are further subcategorised into N- or C-terminally anchored proteins, lipid-anchored proteins (lipoproteins), and LPXTG-anchored proteins. The N-terminally anchored proteins represent the largest group of cell-surface-anchored proteins in lactobacilli and are mainly involved in cell-envelope metabolism, extracellular transport and signal transduction, competence, and protein turnover [35, 63]. Many C-terminally anchored proteins, linked to the cell membrane through C-terminal transmembrane domains, are encoded by lactobacilli, but the function of several of these proteins remains unclear [35]. The lipid-anchored proteins constitute the second largest group of predicted membrane-anchored proteins in lactobacilli, and are involved in transport, adhesion, antibiotic resistance, sensory processes, homeostasis of the cell envelope and secretion, folding and translocation of proteins [35, 64]. The C-region of the signal peptide of these lipoproteins contains the lipobox motif [L-(A/S)-(A/G)-C]. Lipidation followed by cleavage at the N-terminal of the Cys-residue in the lipobox results in the covalent binding of the lipoprotein to the cell membrane through a thioether linkage [65]. LPXTG-anchored proteins or sortase-dependent proteins (SDP) are covalently attached to the PG and reportedly play a crucial role in lactobacilli-host interactions [66]. These proteins typically contain a cleavage site, an LPXTG motif, located in the C-terminal region of the mature domain, followed by a stretch of hydrophobic residues and a positively charged tail [66]. The LPXTG motif is recognised by the sortase (SrtA) enzyme, which cleaves between the T and G residues and then covalently links the threonine carboxyl group to an amino group of the PG cross-bridges [67]. Although SrtA recognises the sequence LPXTG, another sortase, called SrtB in *S. aureus,* has been reported to recognise and process proteins bearing the sequence NPQTN [68]. Recent studies involving cross-linked protein products of SrtA and SrtB indicate that different types of sortases may be able to attach proteins to distinct positions within the cell wall [69].

Noncovalently anchored proteins are bound to the bacterial cell surface through binding domains. Some proteins can also be found anchored to other cell wall proteins through protein-protein interactions, while others are known to reassociate with the cell wall after being secreted, through electrostatic interactions [70].

Many species of lactobacilli display a surface coating made of a crystalline, two-dimensional array of protein or glycoprotein subunits assembled in lattices with different symmetries, also referred to as the S-layer. Lactobacilli S-layer proteins represent up to 10 to 15% of total cell wall proteins. These proteins are highly basic, with stable tertiary structures ranging from 40 to 60 kDa [3]. S-layer proteins are the most prominent glycoproteins in prokaryotes, and although in lactobacilli most S-layer proteins appear to be nonglycosylated, some lactobacilli have glycosylated S-layer proteins that have been identified [71]. S-layer proteins are relevant to cell wall polysaccharide pyruvylation and are noncovalently bound to the underlying PG cell wall, generally through secondary polymers such as LTA, WTA, and neutral polysaccharides [70]. Properties such as adhesion, aggregation, and pathogen inhibition have been related with the occurrence of particular types of S-layers, although S-layer functions in lactobacilli are not just species but also strain specific. Studies indicate that there is a correlation between the different structural and chemical characteristics of the S-layer proteins with the surface properties of lactobacilli [50, 70]. There is ample evidence of S-layer proteins influencing the development of microbial communities as biofilms and therefore, it is likely that S-layer proteins have a role in the interaction of lactobacilli with other microorganisms [72].

Lactobacilli have enzymes with binding domains that help to keep them anchored to the bacterial cell surface. For example, extracellular enzymes such as autolysins display a stretch of 20 amino acids that have conserved multiple tandem repeats of aromatic residues and glycines that anchor to the bacterial cell surface by binding to the choline residues of WTA and LTA [73]. The LysM domain (lysine motif) is found in many extracellular enzymes that are suggested to have a PG binding function and are involved in cell wall metabolism [74]. WXL domain-containing proteins were identified in lactobacilli based on *in silico* analysis [75] and are suggested to interact with the PG layer through their protein C terminus. This domain has also been reported to mediate noncovalent binding between the bacterial cell wall of *Enterococcus faecalis* and other Gram-positive bacteria [76]. SH3b domains have been identified in some lactobacilli and are proposed to be involved in cell wall turnover. They have been suggested to recognise specific sequences within the peptide cross-bridges of the PG, thus targeting and binding to the cell wall [77]. A putative domain composed of three α-helices at the C- or N-terminal of an extracellular protein has been reported in some lactobacilli (*L. plantarum, L. johnsonii, L. casei, L. brevis, L. helveticus,* and *L. gasseri*) and is suggested to be involved in cell wall degradation through binding to the PG [35].

3. Cell Surface Adaptations of Lactobacilli in Response to the Host Environment

The cell envelope is the first target of physicochemical and environmental stress. Lactobacilli encounter several environmental stress factors during their transit through the GIT such as low pH, bile salts, and oxidative and osmotic stress, along with starvation stress. Lactobacilli have developed sophisticated responses and adaptations to survive these stressors. Stress responses of lactobacilli rely on the coordinated expression or suppression of genes that act in concert to improve stress tolerance. These genes can alter cellular processes such as cell division, membrane composition, transport systems, housekeeping, and DNA metabolism and are regulated by factors that can control several genes and sometimes even other regulators. Lactobacilli respond to stress in specific ways dependent on the strain, species, and the type of stress. The coordination of these stress responses is

achieved by the network of regulators that allow the bacterial cell to react and adapt to different stressors.

3.1. Acid and Bile Stress. Survival under acidic conditions is achieved by adapting to low pH through a mechanism called acid tolerance response (ATR). Studies with acid- and bile-resistant variants of *L. acidophilus* suggest that an inducible pre-existing system co-exists with a *de novo* protein synthesis mechanism, which together protect against acid stress [78]. Bile acids are conjugated to glycine or taurine in the liver and enter the intestine where the amino acid may be hydrolysed by bile salt hydrolases (BSH) expressed by bacteria, including lactobacilli. In *L. plantarum*, the capacity to tolerate taurodeoxycholic acid (TDCA) has been attributed to the expression of TDCA hydrolase, but other studies have shown that BSH activity and resistance to bile are unrelated properties in lactobacilli [79, 80]. Many resistance mechanisms resulting in alteration of lactobacilli cell surface structures are common for bile and acid stress [81]. The macromolecules composing the bacterial cell envelope (cell wall and cell membrane) contribute to maintaining the cell integrity under these stress situations. For instance, bile salts and cholesterol have been shown to induce changes in the lipid cell membrane of *L. reuteri* [19] while low pH causes alterations in the fatty acid composition of an oral strain of *L. casei* [20].

Screenings of acid responses and bile salt responses in lactobacilli have identified genes involved in PG biosynthesis and cell envelope functions. Gene expression analysis of *L. acidophilus* identified a high number of genes involved in PG and cell surface protein (e.g., SrtA) biosynthesis that are differentially expressed after bile exposure [82]. In *L. reuteri*, the response to acidic conditions involves the ClpL chaperone, an ATPase with chaperone activity and a putative cell wall-altering esterase. These enzymes are also reported to be induced by bile exposure, further implying common resistance mechanisms for acid and bile stress [83, 84]. Other cell surface structures (LTA, WTA, and EPS) have also been suggested to play roles in proper functioning of cell integrity in acidic conditions and in the presence of bile [85]. EPS biosynthesis also reportedly involves suppression of genes after bile exposure as noted in *L. acidophilus* and *L. reuteri*, although the role of EPS in bile and acid resistance is still unclear [82, 84].

3.2. Oxidative and Osmotic Stress. In addition to acid and bile stress, the survival capacity of lactobacilli to oxidative and osmotic stress in the GIT is important. Oxidative stress that can adversely affect cell fitness is caused by exposure to reactive oxygen species (ROS) resulting from partial oxygen reduction to superoxide anion radicals (O_2), hydroxyl radicals ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2). Polyunsaturated fatty acids are sensitive to ROS attack and the resulting peroxidation of membrane lipids and protein alteration affect cell membrane permeability and osmoregulation [86]. To minimise the damage caused by ROS, lactobacilli counteract ROS generation with the help of enzymes such as catalase, NADH oxidase/peroxidise, and superoxide dismutase (SOD) or nonenzymatic compounds such as ascorbate, glutathione, and Mn^{2+}. Resistance to oxidative stress varies widely between species and strains. Stress handling mechanisms range from preventing formation of ROS, elimination of ROS, and defence against oxidative damage to repair of oxidative damage [87].

The fatty acid composition of the cell membrane of *L. helveticus* has been shown to change underoxidative stress and this was reported to be due to an increased activity of the O_2-consuming fatty acid desaturase system which reduces the free radical damage in the cell [21]. Interestingly, bile stress has also been shown to induce oxidative stress, and studies indicate that the expression of glutathione reductase is influenced by bile treatment [88].

Lactobacilli are often exposed to changes in the osmolarity of their environment which can compromise essential cell functions. Changes in solute concentrations in the environment cause changes in cell turgor pressure which lead to changes in cell volume. To maintain turgor pressure and retain water in the cell, lactobacilli accumulate compatible solutes under hyper-osmotic conditions and release them under hypo-osmotic conditions. In *L. acidophilus*, disruption of the cell division enzyme CdpA caused an increased resistance to bile salts while showing reduced resistance to osmotic stress. Similar effects were shown by the SlpA mutant of *L. acidophilus*, which was more sensitive to osmotic stress while being more resistant to bile. According to these studies, certain components of the cell wall remain uncleaved or cross-linked resulting in an immature structure of the cell wall in the mutant thus altering its phenotype [89, 90]. Studies with *L. alimentarius* showed that when grown under sublethal doses of NaCl, an increased tolerance was observed towards hyper-osmotic conditions or an increased ATR against organic acids. Similar cross-protection was observed when the cells were exposed to sublethal doses of these acids implying that common mechanisms were involved [91].

3.3. Starvation Stress. The capacity to adapt to a specific nutritional environment is important to lactobacilli and ensures their residence time and survival in the GIT. Starvation is one of the most common stresses faced by lactobacilli and bacterial growth leading to nutrient exhaustion, accumulation of fermentation end product (e.g., lactic acid), and subsequent starvation contributes to this stress. Nutrient starvation in lactobacilli has been mainly studied by limiting the supply of carbohydrate, phosphate, and nitrogen. Lactobacilli adapt to these nutritional limitations by either downregulating nucleic acid and protein synthesis and/or protein degradation and amino acid synthesis [92]. Moreover, extreme environmental stress conditions can indirectly provoke starvation by decreasing the activity of transporters resulting in reduced availability of essential nutrients that might be present in the extracellular environment [93]. Nutrient starvation leads to growth arrest, and different lactobacilli have developed different strategies to survive starvation. Modification of cell morphology and cell division at the entry of the stationary phase, resulting in diminished cell size, has been reported in lactobacilli under these conditions [87].

Starvation resistance mechanisms in lactobacilli are diverse as they occupy different niches and do not encounter the same starvation conditions. It is well established that bacteria become more resistant to various types of stresses and develop a general stress-resistant state on entering the stationary phase. Carbohydrate starvation induces increased resistance to many stress conditions. Amino acid catabolism, in particular arginine degradation, plays a role in the enhanced survival of *L. sakei* during stationary phase [94]. In *L. acidophilus*, 16 proteins were reported to be synthesised as a response to starvation, of which 7 were induced by stationary phase while the others in response to low pH [95]. In *L. lactis*, glucose starvation was shown to induce resistance to many stresses (heat, low pH, and oxidative and osmotic stress) [96]. Similarly in *L. bulgaricus*, lactose starvation increased resistance to heat, acid, and bile stress [97]. The regulation of starvation-induced proteins in lactobacilli is still unclear. Although studies indicate a small overlap between stress-specific and starvation regulator genes and many proteins can be commonly induced by more than one stress, only a few proteins are common to all stresses.

4. Lactobacilli and Host Interactions Involving Bacterial Cell Surface Factors

The human GIT represents the first line of defence against bacteria, viruses, fungi, and parasites that can act as pathogens. The GIT epithelium is also associated with indigenous commensal microorganisms that comprise the microbiota. Thus, the epithelium is important for the maintenance of GIT homeostasis in the presence of commensal microorganisms while preventing pathogen invasion [98]. Lactobacilli interact with the intestinal epithelium through several mechanisms that help modulate the immune response of the host, preserve barrier integrity, and maintain microbial balance through exclusion of pathogens by direct antimicrobial activity (production of bacteriocins or inhibitors), competitive exclusion (competing for binding sites), and/or stimulating anti-inflammatory immune responses (Table 1).

4.1. Adherence. Adherence of bacteria to the GIT mucosa is an important factor for colonisation and leads to direct interactions that can result in competitive exclusion of pathogens and the modulation of host response. Adhesive mechanisms of human pathogenic bacteria have been studied extensively through the use of *in vitro* model systems. Human colorectal adenocarcinoma cell lines such as Caco-2 or HT-29 cells, immobilised intestinal mucus and extracellular matrices, quantitative measurements, microscopic enumeration, and immunological detection methods have been used for assessing adhesive mechanisms [120, 121]. However, knowledge of the bacterial cell surface molecules mediating adhesion to the GIT mucosa is still limited. Genomics-based approaches have revealed several bacterial cell-surface-associated proteins that bind to mucus and intestinal cells [17]. Lactobacilli adhesins have been grouped into mucus binding proteins; sortase-dependent proteins; S-layer proteins; proteins mediating adhesion to extracellular matrix (ECM) components of the intestinal epithelial cells; nonprotein adhesins (LTA and EPS).

Intestinal epithelial cells form a barrier between the host and the content of the lumen and are covered by a protective layer of mucus. The mucus layer exists in a dynamic equilibrium, balanced between production, degradation, and physical erosion. It provides bacteria with only a short residence time in the GIT upon adhesion, thereby protecting the host against pathogens and undesirable bacterial colonisation [122]. However, the mucus layer also provides a habitat for commensal bacteria, such as lactobacilli. Adherence of lactobacilli to mucus has been experimentally validated *in vitro* using adhesion assays with probiotic-pretreated intestinal mucus glycoproteins [123], as well as *in vivo* by microscopic analysis of biopsy samples [124]. *Lactobacillus* adhesion to mucus involves mucus binding proteins (Mubs) which in addition to the same domain organisation typical of cell surface proteins (the N- terminal signal peptide and C terminal LPXTG anchoring motif) share a mucus binding domain. Mubs are encoded by Lactobacillales-specific clusters of orthologous protein coding genes (LaCOG) and contain one or more Mub repeats. Proteins containing Mub repeats are abundant in lactobacilli that inhabit the GIT, suggesting that Mub repeat is a functional unit that may be an evolutionary adaptation for survival in the GIT. A database search using the sequence from the extracellular Mub domain of *L. reuteri* [125] and *L. acidophilus* [17], and the lectin-like mannose-specific adhesin (Msa) of *L. plantarum* [103], resulted in the identification of proteins containing multiple Mub domains in several species of lactic acid bacteria (LAB), further suggesting that this domain is a LAB-specific functional unit. Studies with *L. fermentum* BCS87 have helped identify and characterise a 32 KDa surface-associated protein (32-mMubp) that is suggested to mediate adhesion to mucus [114]. The Mub domain consists of a series of amino acid residues, varying in size from 100 to 200 residues per domain [126]. Studies have shown that Mub and Mub-like proteins contribute to mucus binding and autoaggregation, but high genetic heterogeneity among strains results in strain-specific diversity in adhesion to mucus [122].

Some lactobacilli (e.g., *L. rhamnosus* GG) have fimbriae (also called pili) that reportedly enhance adhesion to mucus glycoproteins of the host cells with subsequent colonisation of the GIT [53]. Studies with *L. rhamnosus* GG have shown a mucus binding factor (MBF) with a presumed ancillary involvement in pilus-mediated mucosal adhesion [107]. However, fimbriae of some Gram-positive pathogens were shown to induce pro-inflammatory responses [127], while capsular polysaccharide of *L. rhamnosus* GG was found to shield fimbriae, possibly suppressing pro-inflammatory responses [99]. Such role and possible positive effects of *L. rhamnosus* GG fimbriae are still unclear and need to be validated.

In lactobacilli, a subgroup of surface proteins that contains the LPXTG motif at their C terminal is recognised by SrtA. SrtA cleaves those proteins and anchors the resulting product to PG, thus incorporating these SrtA-dependent proteins on the microbial surface. Although many sortase-dependent proteins are encoded by lactobacilli, the majority have no assigned function. Of the functionally characterised

Table 1: Lactobacilli cell surface factors implicated in microbe-host interactions.

Lactobacillus strain	Mechanism and effect	Cell surface factors	Target cells or host factors	Reference
L. reuteri	Adherence, pathogen inhibition, and enhancement of epithelial barrier function	Mucus binding protein (Mub), collagen binding proteins (CnBP)	Epithelial cells and mucus, collagen	[84, 99, 100]
L. acidophilus	Adherence and aggregation, pathogen inhibition, maintenance of barrier function, and immunomodulation	Mucus binding protein (Mub), fibronectin binding protein (FbpA), S-layer proteins (SlpA), LTA, and EPS	Epithelial cells and mucus, fibronectin, ECM components, and Caco-2 cells	[17, 62, 101, 102]
L. plantarum	Adherence, enhancement of epithelial barrier function, and immunomodulation	Mannose-specific adhesin (Msa), GAPDH	Epithelial cells and mucus, Caco-2 cells	[103–106]
L. rhamnosus	Adherence, protection against pathogen, and antiapoptotic effects on intestinal epithelial cells	Fimbriae, mucus binding factor (MBF)	Mucus glycoproteins, intestinal epithelial cells	[53, 99, 107, 108]
L. salivarius	Adherence	Sortase-dependent protein (LspA)	Intestinal epithelial cells and mucus	[109]
L. crispatus	Adherence, pathogen inhibition, and resistance to acid and bile	S-layer proteins	HeLa cells	[63]
L. brevis	Adherence, protection against stressors (low pH, bile, etc.), and enhancement of barrier function	S-layer proteins (SlpA)	Intestinal epithelial cells	[110, 111]
L. kefir	Aggregation and protection against pathogens and stressors	S-layer proteins	Caco-2/TC-7 cells	[112, 113]
L. fermentum	Adherence	Mucus binding protein (32-mMubp)	mucus	[114]
L. johnsonii	Adherence	LTA, elongation factor Tu (EF-Tu), and heat shock protein (GroEL)	Caco-2 cells, intestinal epithelial cells, and mucus	[102, 115, 116]
L. ruminis	Motility, immunomodulation	Flagellin	Intestinal epithelial cells, HT 29, and Caco-2 cells	[11]
L. casei	Maintenance of barrier function, increased mucus production, and immunomodulation	EPS, sortase-dependent proteins (SrtA)	Caco-2 cells, HT29 macrophages	[117–119]

proteins belonging to this family, three correspond to the mucus adhesins of *L. reuteri* (Mub), *L. plantarum* (Msa), and *L. acidophilus* (Mub). LspA, of *L. salivarius* UCC118, is the fourth characterised sortase-dependent protein that also binds mucus and is known to mediate adhesion of this species to intestinal epithelial cells [109, 128]. Recent studies with *L. casei* BL23 sortases and SrtA mutants suggest that SrtA might be involved in adhesion of this strain to Caco-2 and HT29 cells [117]. Although most sortase-dependent proteins of lactobacilli seem to have mucus-binding capacity, not all of them have affinity to mucus components and the function of putative lactobacilli sortase-dependent proteins remains unclear [16]. Domain analysis and phylogenetic profiling of the extracellular proteins of *L. plantarum* involved in adhesion reported 10 of the 12 identified proteins to contain the LPXTG motif. Their predicted role was adherence to collagen, fibronectin, chitin, or mucus [75]. Of these 12 identified proteins, the role of Msa from *L. plantarum* in adhesion has been experimentally validated, but the roles of the other *in silico* identified putative adhesins are speculative and need *in vitro* and *in vivo* validation.

S-layer proteins form the outermost interacting surface in different species of lactobacilli and have been shown to act as adhesins to epithelial cells and components like mucus and extracellular matrix proteins. The role in adhesion of S-layer proteins of *L. acidophilus* (SlpA), *L. crispatus* (CbsA), and *L. brevis* (SlpA) has been experimentally validated [63, 110, 129]. The removal of the S-layer that reduced bacterial aggregation in *L. acidophilus, L. kefir,* and *L. crispatus* suggests their functional involvement in this process [112, 113]. There is considerable evidence that aggregation directly influences the development of structured microbial communities as

biofilms, and the removal of the S-layer completely abolishes coaggregation, thus suggesting that it is mediated by S-layer proteins. Studies also suggest that S-layer proteins with lectin-like activity interact with glycoproteins and polysaccharides and thus influence interactions of lactobacilli with other microorganisms [113].

Aggregation helps to form a physical barrier thus preventing colonisation by pathogens. Immunoblotting assays show direct interaction between *L. kefir* S-layer proteins and *Salmonella* surface adhesins. Pretreatment of *Salmonella* with purified S-layer proteins has been shown to protect two human intestinal epithelial cell lines, parental Caco-2 and the TC-7 clone, from *Salmonella* invasion, but the protective effect was not observed when *Salmonella* was pretreated with nonaggregative strains [112]. These observations strengthen the theory that coaggregation prevents invasion by *Salmonella* and protects epithelial cell damage. In *L. kefir*, the S-layer also influenced hemagglutinating, but not adhesion to Caco-2 cells, unlike the S-layer of some strains of *L. acidophilus* that are involved in both Caco-2 adhesion and aggregation [100, 112, 113]. In *L. crispatus*, the removal of the S-layer did not affect autoaggregation or hemagglutinating [63], suggesting that the S-layer may not be the only structure involved in these processes and that other covalently bound proteins or molecules such as LTA or lectin-like molecules can mediate adhesion to intestinal epithelial cells.

The extracellular matrix (ECM) is a complex structure surrounding intestinal epithelial cells and is composed of various proteins such as laminin, fibronectin, and collagen. Some lactobacilli can bind to these proteins, thus competing with pathogens that have ligands for the same binding sites [62]. Examples of ECM binding adhesins are the fibronectin-binding protein (FbpA) of *L. acidophilus* and the collagen-binding protein (CnBP) of *L. reuteri* [17, 130]. Pfam domain analysis of CnBP predicted a bacterial extracellular solute-binding domain (PF00497) that was also detected in mucus adhesion promoting protein (MapA), which was found to be a homologue for CnBP. Although MapA reportedly mediates the binding of *L. reuteri* to Caco-2 cells and mucus, database analysis detected no mucus binding proteins, suggesting a role for the extracellular solute-binding domain of MapA in adhesion [16]. Other examples include the previously discussed S-layer proteins.

Lactobacillus adhesion to the GIT has also been shown to involve surface-associated nonprotein factors such as the LTAs and EPS. LTAs contribute to the anionic character of the cell wall and provide hydrophobicity, which in turn influences the adhesiveness of the cell envelope [34]. EPS may contribute to the physicochemical properties of the cell surface by shielding other cell surface adhesins, acting as ligands mediating adhesion and coaggregation [131, 132]. In *L. acidophilus* BG2FO4, carbohydrates on the bacterial cell wall were reported to be partly responsible for adhesion of this strain to Caco-2 cells and to mucus secreted by the mucus producing human adenocarcinoma cell line HT29-MTX cells [101]. In *L. johnsonii*, LTA has been reported to mediate adhesion to Caco-2 cells [18] and in *L. acidophilus*, different types of exopolysaccharides have been shown to influence adhesion to ECM components [62].

Two peculiar examples of cytoplasmic-localised proteins that act as surface-translocated adhesins in lactobacilli are elongation factor Tu (EF-Tu) and the heat shock protein GroEL of *L. johnsonii*. EF-Tu is involved in protein biosynthesis in the cytoplasm but has been reported as surface translocated in many lactobacilli. In *L. johnsonii*, surface translocated EF-Tu fulfills an alternative role of mediating adhesion to intestinal epithelial cells and mucins. GroEL is a mediator of protein folding but when localised at the bacterial surface, it mediates adhesion to human intestinal cells and mucins [115, 116]. No domains or motifs have been found in either protein to account for their translocation across membranes. A cell-surface-associated enzyme GAPDH of *L. plantarum* LA318 has been found to mediate adherence to human colonic cells supposedly by recognising the sugar chains on the mucus and acting as a lectin-like protein [104]. GAPDH is surface localised although it lacks the conventional N-terminal signal sequence or a membrane anchoring motif.

4.2. Maintenance of Epithelial Barrier Function. There is increasing evidence that lactobacilli may have beneficial influences on the intestinal epithelium. The role of lactobacilli in maintaining the intestinal barrier function is achieved by various mechanisms such as inducing mucus production, modulation of cytoskeletal, and tight junction protein phosphorylation, which can enhance tight junction function, immune response, and preventing apoptosis of the intestinal epithelial cells. Enhancement of epithelial barrier integrity by lactobacilli has been observed in both *in vitro* and *in vivo* models. For example, *L. brevis* strengthens epithelial barrier function in healthy rats as assessed by mannitol permeability, with mannitol being used as a probe to study colonic wall permeability [111]. Administration of *L. plantarum* and *L. reuteri* to rats with methotrexate-induced enterocolitis improves bowel barrier function [105]. *L. plantarum* has also been shown to increase epithelial barrier integrity using transepithelial electrical resistance assays as a measure of the integrity of tight junctions between intestinal epithelial cells with Caco-2 cell line as a model [106]. Studies with interleukin-10 gene-deficient (IL-$10^{-/-}$) mice indicate that most of them develop chronic enterocolitis, as IL-10 has been suggested as an essential immunoregulator in the GIT and is a potent suppressor of macrophage and T-cell functions. *Lactobacillus* species have been shown to prevent chronic colitis in IL-$10^{-/-}$ mice [133]. Studies with human intestinal epithelial HT29 cells show that the lipid moiety of LTA from *L. johnsonii* and *L. acidophilus* inhibits *E.coli* and lipopolysaccharide- (LPS-) induced IL-8 production (IL-8 is a chemokine and is a potent promoter of angiogenesis) by epithelial cells thus identifying important bacterial cell surface factors that confer beneficial effects on the GIT [102]. Recent studies with *L. rhamnosus* GG using Caco-2 epithelial cells validate that the lipid chains of LTA are needed for IL-8 mRNA expression and that D-alanine substituents are also important for IL-8 induction in Caco-2 cells [134].

The intestinal epithelial barrier is also affected by alterations in mucus and chloride secretion by epithelial cells.

Mucin forms a physicochemical protective barrier for the underlying intestinal epithelial cells and assists in the prevention of mechanical, chemical, enzymatic, and microbial damage to the intestinal barrier and also restricts microbial invasion following adherence [135]. *In vitro* experiments with selected *Lactobacillus* strains have shown that adherence of enteropathogenic *E. coli* to human intestinal epithelial cells is inhibited by induction of intestinal mucin gene expression [136]. Mucin is known to inhibit bacterial translocation, and studies with *L. casei* LGG showed increased expression levels of mucin genes in a Caco-2 cell model [118]. Expression of mucin genes, induced by lactobacilli, has been shown to be dependent on direct cell contact between *L. plantarum* and intestinal epithelial cells [136].

In addition to mucus production, modulation of tight junction protein expression in epithelial cells is an important factor in preserving epithelial barrier integrity. Tight junction proteins are dynamic structures that bind together epithelial cells at their apical junctions and help maintain barrier integrity. Structural changes in tight junction proteins influence their functionality. Zonula occludens-1 (ZO-1), a tight junction protein, and F-actin, a structural component of the epithelial cell cytoskeleton, are known to play important roles in maintaining cytoskeleton architecture of epithelial cells thus preserving barrier integrity. *L. acidophilus* has been shown to prevent disruption of the distribution of ZO-1 and occludin by *E. coli* and enhance cytoskeletal and tight junction protein structures such as occludin and actinin in intestinal epithelial cells [137]. Lactobacilli also improved barrier function in rats by increasing occludin expression and maintaining epithelial tight junctions [138, 139].

The adherence ability of lactobacilli enables them to compete with pathogenic bacteria for receptors that are expressed on intestinal epithelial cells, thus shielding them from damage caused by pathogenic bacteria and preserving barrier integrity [22, 140]. *L. rhamnosus* R0011 and *L. acidophilus* R0052 inhibit infection of intestinal cells caused by exposure to *E. coli* by reducing bacterial adhesion and cytoskeletal rearrangements [140]. Studies with specific lactobacilli strains show that direct cell contact is needed to induce expression of opioid and cannabinoid receptors in intestinal epithelium mediating analgesic functions in the GIT implying involvement of cell-surface-related effector molecules [141]. Antiapoptotic effect of *L. rhamnosus* GG in intestinal epithelial cells is also dependent on direct cell contact [102]. The activation of the antiapoptotic Akt/protein kinase B and inhibition of the activation of proapoptotic p38/mitogen-activated protein kinase by cytokines were suggested to prevent apoptosis in the intestinal epithelial cells [108].

4.3. Immunomodulation. Lactobacilli are able to modulate immune responses of the host by interaction with the GIT mucosa. Bacterial surfaces exhibit characteristic features known as microbe-associated molecular patterns (MAMP), which are usually cell wall components, such as LPS, PG, LTA, and WTA, but can also be lipids, lipoproteins, proteins, and nucleic acids [142, 143]. MAMPs are recognised by various pattern recognition receptors (PRR) that are expressed by many cell types including immune cells, intestinal epithelial cells, and nonimmune cells. Recognition of these MAMPs by PRRs induces a signalling cascade that can result in the production of cytokines, chemokines, and other effector molecules thus activating the innate immune response in the host. PRRs include toll-like receptors (TLR), nucleotide oligomerization domain (NOD)-like receptors (NLR), and C-type lectin receptors (CLR). Of these, TLRs and NLRs are well-characterised receptors of the host immune system that are known to interact with bacterial cell surface components like the LTA and PG [64]. TLR signalling pathways involve the recruitment of adaptors such as myeloid differentiation primary response gene 88 (MyD88), which in turn activates the mitogen-activated protein kinase (MAPK) pathway and the nuclear factor κB (NF-κB) pathway signalling cascades [144]. Similarly, NOD receptors also activate the MAPK pathway and NF-κB pathway signalling cascades. Activation and translocation of NF-κB result in the transcription of numerous genes that regulate inflammatory responses. Genes regulated by NF-κB include those encoding cytokines such as interleukins (ILs) and tumour necrosis factors (TNFs). These changes in cytokine production can result in dendritic cell (DC) maturation and activation, which in turn modulates the activation and differentiation of T cells [145, 146]. The specific interactions of MAMPs with PRRs and the subsequent induction of signalling pathways depend on the microorganism and the reactivity of the host, which together play major roles in maintaining the functionality and homeostasis of the intestinal epithelial barrier.

Lactobacilli cell wall components such as LTA and lipoproteins are recognised by TLR2 in combination with TLR6, leading to activation of NF-κB. The two lipid chains of LTA have to be exposed to mediate the interaction with the lipid-binding pocket of TLR2 implying that LTAs may not be key PRR ligands for intestinal epithelial cells [147]. WTA and LTA also bind to macrophage scavenger receptors such as SRA, a type I macrophage scavenger receptor that recognises LTAs, thus contributing to immune signalling [148]. LTA and S-layer protein A (SlpA) interact with DC-specific intercellular adhesion molecule-grabbing nonintegrin (DC-SIGN) on DC to induce cytokine release and T-cell maturation. Activation of DC-SIGN by some strains of lactobacilli affects maturation of DCs, which reduces their capacity to induce IL-10-producing regulatory T-cell responses against pathogens [149]. Glycosylation of SlpA might be necessary for its interaction with DC-SIGN but needs to be validated as DC-SIGN is known to interact with glycosylated ligands of pathogens influencing host response to microorganisms [150]. EPS and other cell wall polysaccharides can be recognised by C-type lectin receptors (CLR) that are present on macrophages and DC. In *L. casei* Shirota, suppression of pro-inflammatory responses in macrophages is mediated by EPS thus indicating an immune suppressive role of cell wall polysaccharides [119]. The ability of lactobacilli to induce host cytokine responses in immune cells can be strikingly different depending on both species and strain. Studies of DC responses to 42 *L. plantarum* strains indicate that cytokines produced can vary from strain to strain, and different strains of the

same species can have distinct pro-inflammatory and anti-inflammatory profiles, suggesting that multiple factors can influence immune phenotype [151]. Studies with *L. ruminis* show that some species of lactobacilli display flagella which act as MAMPs that are recognised by the TLR5 of the host and are suggested to activate the NF-κB pathway signalling in epithelial and immune cells of the host [11].

5. Strain Specificity and Cell Surface Factors

An understanding of the roles played by bacterial MAMPs (LTA, WTA, PG, and EPS) in strain-specific effects observed in lactobacilli is still developing. Although MAMPs have a similar basic structure in conserved classes of bacterial macromolecules, different microorganisms can display subtle structural variations between MAMPs located on their cell walls. These variations can mean that a macromolecule from one species or strain can act as an agonist for a PRR, whereas a similar macromolecule from another species or strain acts as an antagonist for the same PRR [152]. Studies indicate that adherence characteristics (a major factor in the colonising potential of commensal bacteria) are influenced by cell wall structure and show pronounced variation among strains [9]. Strain specificity is undoubtedly linked to the variability and biochemical complexity of lactobacilli ligands and MAMPs as seen in the substitution levels of TAs, the variable backbone alditol compositions of the WTA, and the modifications of the PG of the cell wall [153]. These modifications in the structure of PG can affect the physiology of the bacterial cell wall by increased sensitivity to autolysis, resistance to lysozyme, and hydrophobicity of the cell envelope which in turn affects recognition by host receptors and bacterial adhesion [33, 34].

For example, *L. salivarius* str. Ls33 protects against chemically induced colitis in mice through the interaction of muramyl dipeptides present in its PG with NLR of the intestinal epithelial cells. However, this protective effect is not observed for *L. acidophilus* str. NCFM, as variation in the PG composition of this strain blocks the nucleotide binding domain and leucine-rich repeat containing family (NLR) signalling pathway, which activates the MAPK and NF-κB pathways thereby hindering the activation of host defence mechanisms [154]. Another example of strain-specific characteristics imparted by variation in PG composition is observed in several lactobacilli, where resistance to vancomycin (a glycopeptides antibiotic) was shown to be the result of a replacement of the C-terminal D-alanine residue of MurNAc-pentapeptide by D-lactate [155]. This illustrates the importance of the variable biochemistry of MAMPs such as PG to strain or species specificity. In addition, milieu-dependent switching between the multiple variants of cell wall polymers and/or TAs adds to strain variation in lactobacilli. Studies with mutants of *Lactobacillus* strains that produce alternative LTA variants suggest that modifications to the LTA backbone can alter cytokine induction capacity thus increasing anti-inflammatory immune modulation [156, 157]. Studies with dairy-isolated strains of *L. delbrueckii* showed anti-inflammatory effects *in vitro*, but the extent of these effects varied between strains [158]. These effects are suggested to be linked to the bacterial surface exposed proteins. An interesting observation is that *L. delbrueckii* subspecies bulgaricus 1489 shows poor adherence to Caco-2 epithelial cells implying lack of surface factors in this strain that may be involved in adherence [159]. The high diversity of cell surface components found in lactobacilli adds to strain variation and is reflected in the ecological versatility observed in lactobacilli. Chain length variation, subcellular localisation, and interactions of these polymers most likely contribute to strain-specific characteristics and are still being validated experimentally [50, 160].

6. Conclusion

The cell wall is a dynamic entity and plays an essential role in many aspects of the physiology and functioning of lactobacilli. It is where interaction with the bacterial environment occurs, which influences communication and adaptation to host-derived factors encountered in the GIT. Environmental stressors have been shown to affect the cell surface architecture by influencing PG biosynthesis, expression of EPS and cell surface proteins, and LTA decoration with D-alanine residues. Lactobacilli display considerable variation in their cell surface properties, through adaptations which undoubtedly are important for the functioning and survival of these bacteria in the GIT. The increasing possibilities of genomics-based approaches and mutant analyses have resulted in the identification of several effector molecules of lactobacilli. These effector molecules are proposed to be involved in direct interactions with host epithelial or immune cells and many of these effector molecules are components of the cell wall itself [35]. Considering the complexity of host-lactobacilli interactions involving host-cell signalling and regulation pathways, it seems unlikely that single-effector molecules regulate the entire host response. These molecules probably have an expanded repertoire in addition to playing crucial roles as building blocks of the bacterial cell wall [156]. Knowledge of the molecular mechanisms underlying the physiological characteristics of lactobacilli, and identification and validation of effector molecules complemented with parallel studies for their corresponding receptors in the host cells, can strengthen the concept of strain specificity and contributes to the development of strains with enhanced health benefits.

Acknowledgments

The authors thank Pauline Hunt for drawing Figure 1 and Dragana Jankovic and Wayne Young for proofreading the paper. R. Sengupta, supported by a Massey Doctoral Fellowship and a Ph.D. stipend from AgResearch as part of the Centre of Research Excellence (CORE) funding from the Riddet Institute, conducted the literature search and wrote the review. All authors designed the approach, commented, edited, and approved the paper and are responsible for the final version of the paper.

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Hindawi Publishing Corporation
Mediators of Inflammation
Volume 2013, Article ID 258209, 11 pages
http://dx.doi.org/10.1155/2013/258209

Review Article

Current Perspectives in NSAID-Induced Gastropathy

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Received 30 December 2012; Accepted 14 February 2013

Academic Editor: Eduardo Arranz

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most highly prescribed drugs in the world. Their analgesic, anti-inflammatory, and antipyretic actions may be beneficial; however, they are associated with severe side effects including gastrointestinal injury and peptic ulceration. Though several approaches for limiting these side effects have been adopted, like the use of COX-2 specific drugs, comedication of acid suppressants like proton pump inhibitors and prostaglandin analogs, these alternatives have limitations in terms of efficacy and side effects. In this paper, the mechanism of action of NSAIDs and their critical gastrointestinal complications have been reviewed. This paper also provides the information on different preventive measures prescribed to minimize such adverse effects and analyses the new suggested strategies for development of novel drugs to maintain the anti-inflammatory functions of NSAIDs along with effective gastrointestinal protection.

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most well recognized drugs worldwide for the treatment of pain, inflammation, and fever [1–4]. NSAIDs are commonly administered for treatment against inflammatory diseases, rheumatoid arthritis, osteoarthritis, dysmenorrhea, and ischemic cerebrovascular disorders [5]. Use of these drugs in certain types of cancer treatment has also been reported recently [6, 7]. These drugs inhibit prostaglandin biosynthesis and produce their therapeutic effects [8]. However, long-term administration of NSAIDs causes adverse gastrointestinal (GI) symptoms including mucosal lesions, bleeding, peptic ulcer, and inflammation in intestine leading to perforation, strictures in small and large intestines, leading to chronic problems [9–11]. Some of the adverse effects of NSAIDs may be asymptotic, but in many cases there are reports of life-threatening incidents [10].

Such rampant use of NSAIDs requires a focused approach to avoid the possible side effects arising from their use. In this regard, several prevention methods have been used. These are based on usage of a new class of NSAIDs which does not inhibit a specific gastroprotective cascade or coprescription with proton pump inhibitors (PPIs) and prostaglandin analogues to suppress acid secretion [12–15]. However, these methods also have limited potency because of their additional cardiovascular effects [16–19].

Several clinical practice guidelines have proposed different approaches for controlling the GI complications associated with NSAIDs. A number of strategies have been recommended by American College of Gastroenterology to decrease NSAID-induced GI damage including use of selective cyclooxygenase-2 inhibitors, coadministration of gastroprotective agents like misoprostol, PPIs, or histamine-2 receptor antagonists [20]. These strategies are based on multiple risk factors associated with NSAID-induced GI complications including age of the patient, simultaneous medications, prior medical history, and *Helicobacter pylori* infection. The risk of GI bleeding enhances when patients already on antiplatelet therapy using thienopyridines, like clopidogrel, are coprescribed with NSAIDs to reduce adverse cardiovascular events [21]. In 2008, the Clinical Expert Consensus Document prepared by the American College of Cardiology, American College of Gastroenterology and American Heart Association has set the guidelines for reducing GI injury in patients undergoing antiplatelet therapy along with NSAIDs [22]. As per the guidelines, PPIs were recommended for gastroprotective therapy to the patients on thienopyridines

Table 1: Classification of NSAIDs.

Types	Chemical composition	Common NSAIDs
Salicylates	Derivatives of 2-hydroxybenzoic acid (salicylic acid)	Aspirin, diflunisal, and salsalate
Propionic acid derivatives or "profens"	Derivatives of arylacetic acids	Ibuprofen, dexibuprofen, ketoprofen, dexketoprofen, naproxen, fenoprofen, flurbiprofen, oxaprozin, and loxoprofen
Acetic acid derivatives	Derivatives of acetic acids	Indomethacin, diclofenac, nabumetone, tolmetin, sulindac, etodolac, and ketorolac
Enolic acid derivatives or oxicams	Derivatives of 4-hydroxy benzothiazine heterocycle	Piroxicam, isoxicam, meloxicam, tenoxicam, droxicam, and lornoxicam
Fenamic acid derivatives or fenamates	Derivatives of anthranilic acid	Mefenamic acid, flufenamic acid, tolfenamic acid, and meclofenamic acid
Phenylpyrazolones	Derivatives of 1-aryl-3,5-pyrazolidinedione	Phenylbutazone, oxyphenbutazone
COX-2 selective inhibitors	Diaryl-5-membered heterocycles	Celecoxib, rofecoxib, and valdecoxib
Anilides and sulphoanilides	Acetamides of aniline with or without a 4-hydroxy or 4-alkoxy group	Acetaminophen, phenacetin, and nimesulide

and NSAIDs. However, based on some reports suggesting possible interactions between PPIs and thienopyridines [23, 24], the expert guidelines have been further updated in 2010 [25]. The Expert Consensus Report has been prepared taking into account the potential risks and benefits from simultaneous intake of PPIs and thienopyridines. Prescription of PPIs is only recommended for patients on antiplatelet therapy who are at risk for gastrointestinal complications [25].

Till now, there is no effective treatment yet developed for addressing the NSAID-related gastric damage. Identification of the protective factors for gastrointestinal complications associated with NSAIDs still poses a serious challenge. This paper outlines the mechanism of NSAIDs action with their prevalent side effects and provides an insight into the new advances in rational use of NSAIDs for prevention of possible side effects without any compromise on the analgesic properties of the NSAIDs.

2. Properties of NSAIDs

NSAIDs possess certain common pharmacologic properties. Mostly they are organic acids with pKa in the range of 3–5 [5]. In general, they contain an acidic group mostly carboxylic acids or enols. The acidic moiety is essential for COX inhibitory activity and is linked to a planar, aromatic group. The latter is also connected to a lipophilic part through a polar group. The NSAIDs are classified into different groups based on their chemical structure and mechanism of action (Table 1). NSAIDs are generally chiral molecules (except diclofenac), but mostly a single enantiomer is pharmacologically active [26].

3. Mechanism of Anti-Inflammatory Action of NSAIDs

The mechanism of action of NSAIDs was first defined in early seventies and is based on inhibition of prostaglandin (PG) synthesis [8]. PG is one of the main mediators of inflammation, pain, and fever and is synthesized from arachidonic acid. The reaction is catalyzed by the enzyme, cyclooxygenase (COX) earlier referred to as PGH synthase [5]. NSAIDs block PG formation by binding and inhibiting COX (Figure 1).

The analgesic activity of the NSAIDs has been demonstrated to be due to the interference of PGE1 and PGF2 in animal pain models [27, 28]. It has also been observed that NSAIDs are effective against pain because of their ability to inhibit PG-mediated cerebral vascular vasodilation [29, 30]. Several studies have shown that the antipyretic action of NSAIDs is via inhibition of PGE2 synthesis in and near the preoptic hypothalamic area in circumventricular organs [31–33].

4. Mechanism of NSAID-Induced GI Injury

There are mainly three different mechanisms of NSAID-induced GI complications: inhibition of enzyme COX-1 and gastroprotective PG, membrane permeabilization, and production of additional proinflammatory mediators (Figure 2).

4.1. Inhibition of COX-1 and Gastroprotective PG. There are two isoforms of COX, COX-1 and COX-2, which have different functions [34]. COX-1 is constitutively expressed and is responsible for the normal physiological protection of gastric mucosa. It is responsible for the synthesis of prostaglandins, which protects the stomach lining from the secreted acid, maintains blood flow in gastric mucosa, and produces bicarbonate [35, 36]. The other isoform, COX-2, is triggered by cell damage, various proinflammatory cytokines, and tumor-derived factors [37, 38]. NSAID-induced gastropathy is caused mainly by inhibition of COX-1 by NSAIDs [39–41].

4.2. Membrane Permeabilization. NSAIDs also have a direct cytotoxic effect on gastric mucosal cell causing lesions and

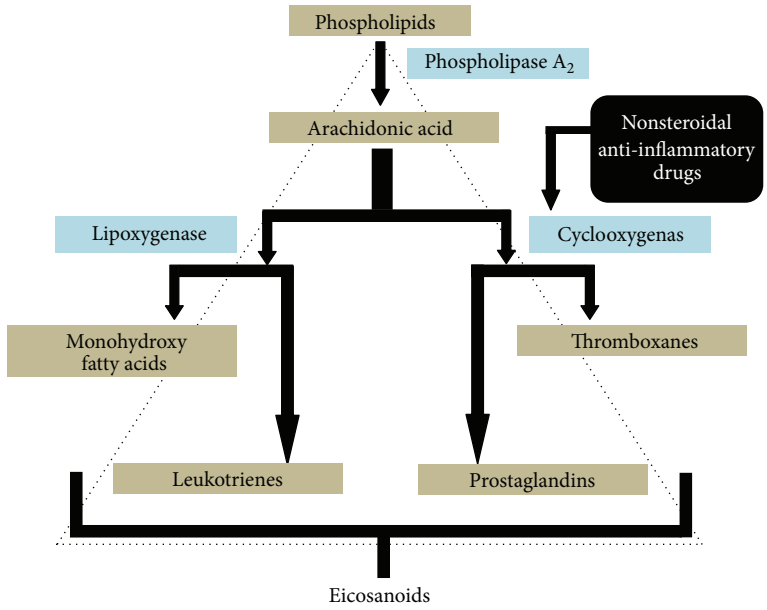

Figure 1: Schematic representation of inhibition of prostaglandin synthesis by NSAIDs.

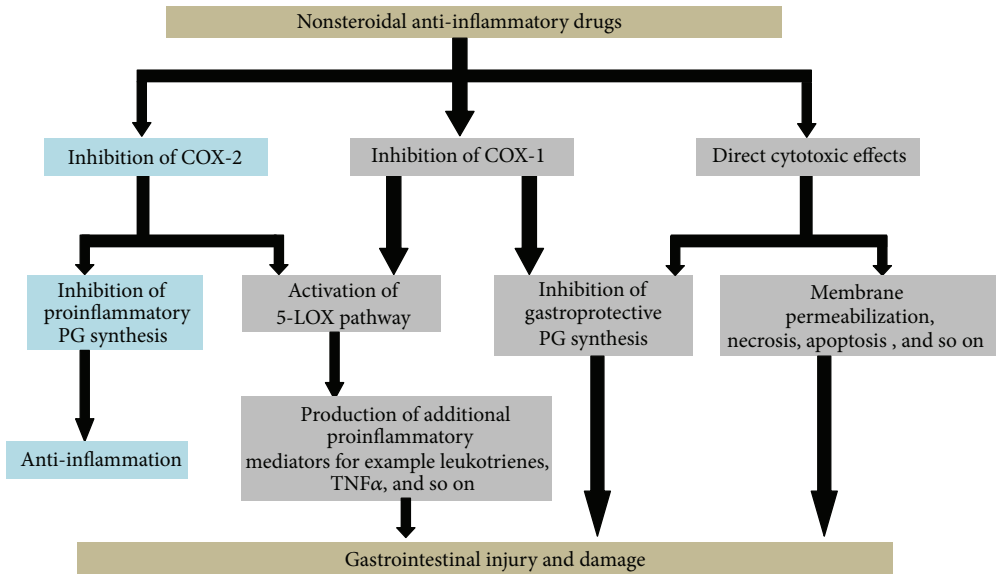

Figure 2: Schematic diagram of mechanism of NSAID-induced gastrointestinal injury and damage.

injury [42, 43]. Some studies have shown that direct cytotoxicity is independent of the inhibition of COX activity [44]. Topical damage of this kind has been observed in the case of acidic NSAIDs like aspirin resulting in accumulation of ionized NSAID, a phenomenon called "ion trapping" [45]. It is suggested that NSAIDs cause membrane permeabilization leading to disruption of epithelial barrier [46]. NSAIDs were also able to induce both necrosis and apoptosis in gastric mucosal cells [47].

4.3. Production of Additional Proinflammatory Mediators. Inhibition of PG synthesis by NSAIDs leads to simultaneous activation of the lipoxygenase pathway and increased synthesis of leukotrienes (Figure 1) [48–50]. Leukotrienes cause inflammation and tissue ischaemia leading to gastric mucosal injury [51, 52]. Along with this, there is also enhanced production of proinflammatory mediators such as tumour necrosis factors [53]. This further leads to occlusion of gastric microvessels leading to reduced gastric blood flow and release

Table 2: . Strategies to prevent NSAID-induced gastrointestinal injury.

Treatment procedure	Mechanism	Action
Gastroprotective drugs		
(i) PG analogues	Replacement of PG	Reduces ulceration and other GI damages Cannot prevent dyspepsia
(ii) Acid suppressants like proton pump inhibitors	Increase of intragastric pH	Decreases dyspepsia, ulceration, and associated damages Not suitable for patients with *H. pylori* infections
Selective COX-2 inhibitors	Does not inhibit COX-1, and hence synthesis of gastroprotective PG is maintained	Reduces dyspepsia, reverses gastroduodenal ulcers, and prevents other GI damages Associated with prothrombotic events and enhances cardiovascular risks
NSAID prodrugs like NO-NSAIDs	Release of NO maintains microvascular integrity	Reduces GI damage, has antithrombotic effects
Inhibitors of COX and 5-LOX	Blocks formation of leukotrienes and other proinflammatory mediators	Maintains gastroprotection and reduces GI damage
Role of lactoferrin	Structural studies suggest binding of C-terminal lobe of lactoferrin with NSAIDs and sequestration of unwanted NSAIDs	Animal studies indicate reversal of gastric bleeding and inhibition of myeloperoxidase formation

of oxygen-derived-free radicals [54]. Free oxygen radicals react with poly unsaturated fatty acids of the mucosa leading to lipid peroxidation and tissue damage [54].

5. Current Therapies for Prevention of Gastric Damage

Several approaches have been adopted for addressing the prevention and cure of the possible side-effects produced by the NSAIDs in the gut. Some of these strategies are routinely prescribed to the patients administering NSAIDs. Presently, the most common protective strategies adopted are (1) combination therapy of NSAIDs with gastroprotective agents and (2) use of selective COX-2 inhibitors (Table 2).

5.1. Combination Therapy of NSAIDs with Gastroprotective Agents

5.1.1. PG Analogues. PG analogues are prescribed with NSAIDs for replenishment of PG which is inhibited by NSAIDs. Misoprostol, a widely used PG analogue, was found to reduce NSAID-induced gastroduodenal ulceration considerably [12]. However, it fails to prevent the reduction of dyspepsia and other GI adverse effects and hence has a limited efficiency [55, 56]. Recently it has been reported that the single-tablet formulations of diclofenac and misoprostol which have been found to be effective in arthritis and in reducing the NSAID-induced gastropathy [57].

5.1.2. Acid Suppressants. Acid increases NSAID-induced mucosal injury and gastric absorption of acidic NSAIDs. H2-receptor antagonists and proton pump inhibitors (PPIs) are most commonly used because they not only reduce acid secretion but also enhance gastric pH and have a role in scavenging-free radicals [58, 59].

H2-receptor antagonists were the first drugs to be used as a prevention mechanism against NSAID-induced peptic ulcers [60]. They were found to be effective against gastric ulceration to a considerable extent [61]. However, no signs of improvement were observed in cases of gastric bleeding, [62] and hence, these drugs are no longer recommended presently.

PPIs are effective in terms of acid suppression and prevention of peptic ulcers when coadministered with the NSAIDs. PPIs are generally prescribed for long-term use since they do not show any significant risk of any associated effects [63, 64]. Omeprazole, a PPI widely prescribed with NSAIDs, can specifically block the parietal cell H^+/K^+-ATPase, thereby significantly inhibiting the gastric acid secretion [65]. Omeprazole was followed by other PPIs like lansoprazole, pantoprazole, rabeprazole, and so forth [66]. Another report has indicated the formulation of lansoprazole, in the form of fast disintegrating tablet to reduce GI injury [67]. Esomeprazole, the S-isomer of omeprazole, has been found to provide a sustained gastric acid control as compared to other PPIs [68]. Considerable reduction of adverse GI symptoms has been observed in patients prescribed with esomeprazole along with NSAIDs or selective COX-2 inhibitors [69, 70]. The first NSAID/PPI single tablet formulation to be approved is ketoprofen/omeprazole modified release capsules [71].

Dual antiplatelet therapy with thienopyridine like clopidogrel and NSAID like aspirin is prescribed to decrease adverse cardiac events in patients suffering from acute coronary syndromes or placement of an intracoronary stent [72, 73], but they are associated with high risks of GI bleeding [21]. PPIs are found to be effective in reducing the risk of GI bleeding in such patients [23]. Clopidogrel is a prodrug that is transformed in vivo to an active metabolite by the cytochrome P450 enzyme system [74]. However, some

reports have suggested that PPIs interfere with clopidogrel to impair platelet function [23, 24, 75]. PPIs possibly inhibit hepatic cytochrome P450 2C19 (CYP2C19) isoenzyme preventing the conversion of clopidogrel into its active metabolite. It has been reported that concurrent use of clopidogrel plus a PPI was associated with a significant increase in risk of an adverse cardiovascular event in patients with acute chronic syndrome [76, 77]. In contrast to this, some other trials did not find any enhanced risk of adverse effects of the use of PPI in combination with clopidogrel [78, 79]. Thus, though routine use of a PPI is not recommended for patients in general, but it is coprescribed in patients with potential risk of GI bleeding [25, 80].

The main drawback of PPIs is that they are less effective against mucosal injury in more distal parts of the intestine like NSAID-induced colonopathy [81]. Moreover, these agents are not prescribed to patients suffering from *H. pylori* infection because of occurrence of corpus gastritis [82].

5.2. Selective COX-2 Inhibitors. Selective COX-2 inhibitors, as the name suggests, are a group of drugs which selectively inhibit the COX-2 inhibitors, thus maintaining the anti-inflammatory properties of NSAIDs, yet retaining the gastroprotective action elicited by COX-1 pathway [83–85]. By far, celecoxib and rofecoxib stand out as the most effective COX-2 inhibitors and show efficacy over nonselective NSAIDs in regard to GI complications including mucosal lesions and other adverse GI symptoms [86, 87].

Several classes of COX-2-selective inhibitors have been identified, including the diarylheterocyclics (or tricyclics), acidic sulfonamides, and 2,6-ditert-butyl phenols, as well as the derivatives of the nonselective inhibitors zomepirac, indomethacin, piroxicam, and aspirin [88–90]. Celecoxib was first identified in 1997 and approved in 1998 [91, 92]. It has been found to preferentially inhibit COX-2 but exhibited the anti-inflammatory, antipyretic, and analgesic activities of NSAIDs [86, 93, 94]. Rofecoxib launched in 1999 was found to be effective in the treatment of osteoarthritis and pain [87, 95–97]. Similarly, nimesulide was highly selective against COX-2, so that at concentrations attained in vivo, while it had no substantial effect on COX-1, it suppressed COX-2 significantly [98].

Though COX-2 inhibitors decrease the GI toxicity to a considerable amount, there is an associated risk of cardiovascular complications due to myocardial infarction and thrombosis associated with their use [99–104]. COX-2 inhibitors have been demonstrated to inhibit the production of vascular prostacyclin, which has vasodilatory effects, and inhibits platelet aggregation unlike nonselective NSAIDs [105, 106]. Longer term gastrointestinal data from the celecoxib study (CLASS) and cardiovascular adverse event data from the rofecoxib study (VIGOR) have questioned the usage of these new drugs [86, 87, 107]. Some of these potent drugs have even been withdrawn [108].

6. Recent Advances in NSAID Treatments

6.1. Prodrugs of NSAIDs. NSAID prodrugs are potential agents for enhancing the antioxidant activity, water solubility and dissolution, release of nitric oxide and hydrogen sulfide, site-specific targeting and delivery, and inhibiting anticholinergic and acetylcholinesterase activity [109–113].

6.1.1. Nitric Oxide Releasing NSAIDs. It has been observed that nitric oxide (NO) imparts gastroprotection by increasing blood flow, mucus production, and bicarbonate secretion in the gastric mucosa [114–116]. NO formed by the action of nitric oxide synthase increases mucus and bicarbonate secretion as well as microcirculation and decreases neutrophil-endothelial adherence [117]. This led to the development of new therapeutic drugs: nitric oxide releasing NSAIDs (NO-NSAIDs) [118]. These drugs are developed by modifying NSAIDs esterified to a NO releasing moiety. Animal studies have demonstrated that NO-NSAIDs do not affect the gastroduodenal mucosa [119–121]. NO naproxen has been also been found to enhance anti-inflammatory and antinociceptive efficacy [122]. NO aspirin has been found to impart an increased antithrombotic potency compared with conventional aspirin [123, 124].

6.1.2. Hydrogen Sulfide Releasing NSAID. Hydrogen sulfide (H2S) also exerts its gastroprotective effects and reverses preexisting ulcers. Derivatives of naproxen, diclofenac, and indomethacin which can release H2S have been reported [125–128]. Phosphatidylcholine-associated NSAIDs as well as NO- and H2S-releasing NSAIDs are under extensive preclinical testing for their influence on NSAID induced GI toxicity [129, 130].

Further studies are in progress to develop promising new NSAIDs imparting total GI (upper and lower GI tracts) protection and without cardiovascular toxicity. Recently a diclofenac prodrug, 1-(2,6-dichlorophenyl)indolin-2-one, has been demonstrated with anti-inflammatory properties that can decrease PGE2 levels, COX-2 expression, and ulceration [131]. In yet another experiment, it was observed that ibuprofen R(−) isomer is a better agent in preventing GI toxicity than S(+) isomer because of short plasma-elimination half-life, its limited ability to inhibit PG synthesis. The R(−) isomer is then converted in the body to the S(+) isomer after absorption in the GI tract [132].

6.2. Simultaneous Inhibition of COX and 5-LOX. NSAID-induced inhibition of COX also results in increased production of leukotrienes, one of the potent mediators of inflammation [49–51]. Recent approach for addressing NSAID-induced GI injury is by development of inhibitors of COX/5-LOX simultaneously [133, 134]. Licofelone ([2,2-dimethyl-6-(4-chloropheny-7-phenyl-2,3-dihydro-1H-pyrrazoline-5-yl]acetic acid) has been identified as one of the most convincing compounds in this group [135]. Licofelone imparts significant analgesic and anti-inflammatory effects without any GI side-effects as observed in animal models [136]. It significantly improved indomethacin-induced gastric ulceration and prevented NSAID-induced increase in leukotriene levels in gastric mucosa [137]. The preclinical evaluation has suggested that licofelone has a promising pharmacodynamic effect [138]. Further clinical trials are in

progress in osteoarthritis patients [139]. Licofelone has also been found to be effective because of its antithrombotic and platelet aggregation inhibiting functions [140]. Earlier to this, benoxaprofen identified as a dual COX/5-LOX inhibitor was withdrawn because it was found to induce severe hepatic and other toxicities [141].

6.3. Role of Lactoferrin in Reducing NSAID-Induced Gut Damage. Some preliminary reports have shown that bovine colostrum has the ability to prevent NSAID-induced gastric ulcers [142, 143]. Further studies have demonstrated the role of recombinant human lactoferrin in decreasing acute NSAID-induced GI bleeding and reduction of gastric ulcers [144, 145]. Recent reports also suggest that C-lobe of lactoferrin, which is resistant to enzymatic degradation [146], has excellent sequestering property for such class of drugs [147]. Further reports have shown that C-lobe of lactoferrin can also bind to COX-2-specific drugs and produce observable effects against gastric inflammation and bleeding [148]. Experiments on rodent model suggest that C-lobe of lactoferrin considerably diminishes the NSAID-induced GI bleeding and inflammation in case of conventional NSAIDs as well as COX-2-specific NSAIDs [147]. In this regard, development of such new molecules that can sequester the unbound drug molecules is essential for addressing the NSAID-related GI damage.

7. Conclusions

The therapeutic effects of NSAIDs have made these drugs extremely popular against inflammatory disorders for the past several decades. However, these drugs suffer from serious drawbacks in cases of long-term administration, including severe GI complications. Several strategies have been adapted to control the critical side-effects. Though, these treatments are effective to some extent, but most of them are also associated with other risks.

Thus, there is no drug yet formulated that can avert the potential side-effects completely. There is an urgent need to develop novel therapeutic agents to make the use of NSAIDs safer. New measures of treatments such as dual COX/5-LOX inhibitors, prodrugs of NSAIDs, or agents that can effectively sequester the unbound NSAIDs without interfering their efficacy can prove to be superior strategies compared to the existing ones.

Acknowledgments

The authors acknowledge financial support from the Department of Biotechnology (DBT), New Delhi. T. P. Singh thanks the Department of Biotechnology (DBT), for the award of Distinguished Biotechnology Research Professorship awarded to him. M. Sinha thanks Department of Science and Technology (DST), Ministry of Science and Technology, New Delhi, L. Gautam thanks Council of Scientific and Industrial Research (CSIR), New Delhi, and P. K. Shukla thanks Indian Council of Medical Research (ICMR), New Delhi, for the award of fellowships.

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